A KINETIC ANALYSIS OF CATION INTERACTIONS IN THE PYRUVIC PHOSPHOFERASE SYSTEM OF RABBIT ERYTHROCYTES

by

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UNIVERSITY OF ALBERTA

SCHOOL OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the School of Graduate Studies for acceptance, a thesis entitled "A Kinetic Analysis of Cation Interactions in the Pyruvic Phosphoferase System of Rabbit Erythrocytes" submitted by Thomas Evan Webb, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The nature of the mechanism underlying cation transport and its relation to the problem of maintenance of a normal K/Na gradient in erythrocytes on storage have been subject to extensive investigation. The general physiological phenomenon of cation antagonism has also been studied using various approaches. The failure to find adequate answers to either problem has prompted research workers to investigate cation interactions at the enzyme level. Such an approach undoubtedly entails the problem of elucidating the mechanism by which cations activate and inhibit enzymes.

One of the most useful enzymes from the standpoint of studying cation interactions is pyruvic phosphoferase (PPFase) which facilitates the transfer of high energy phosphate from phospho(enol) pyruvic acid (PEPA) to adenosine diphosphate (ADP). PPFase has an absolute requirement for a univalent cation such as K^+ (replaceable by NH_4^+ or Rb^+) and a divalent cation such as Mg^{++} (replaceable by Mn^{++} or Co^{++}). It is inhibited by Na^+, Li^+, and Ca^{++}.

Cation interactions have been studied in the PPFase system by use of the optical assay method. The PPFase system was coupled to the lactic dehydrogenase system, and the oxidation of reduced diphosphopyridine nucleotide (DPNH), which has an absorption maximum at 340 mμ, was followed spectrophotometrically.

Cation interactions have been studied by varying only two parameters of the system at any one time, while the others are held constant. The source of the enzyme has been exclusively the dialyzed hemolysate of rabbit erythrocytes.

Activation studies indicate that K^+, although obligatory in the PPFase system, serves in the capacity of a noncoupling activator in respect to both substrates, PEPA and ADP.

Graphical analysis indicates that Mg^{++} must be attached to the enzyme before PEPA will couple with the enzyme. It is referred to as a coupling activator of the first type. The results with the other substrate, ADP, suggests noncoupling activation by Mg^{++}.

Inhibition by excess K^+ appears to be due to coupling with PEPA. Inhibition by excess Mg^{++}, on the other hand, appears to be due to competition of a second ion of Mg++ with PEPA for a center in the active locus of the enzyme.

Na^+ exerts its main inhibitory action by coupling with PEPA. The inhibitory action of Na^+ does not seem to be related in any way to the participation of ADP in this system. Na^+ inhibition can be relieved to some extent by increasing the (Mg^{++}), although graphical analysis suggests that Na^+ inhibition is of a noncompetitive nature with respect to both Mg^{++} and K^+.

Ca^{++} appears to exert its inhibitory action by direct interaction with the enzyme, since it shows true competitive inhibition with respect to Mg^{++}. Although graphical analysis suggests that Ca^{++} inhibition is noncompetitive with respect to K^+, the latter ion can relieve, in part, inhibition at lower (Ca^{++}). Increasing (ADP) also decreases Ca^{++} inhibition to some extent.

The Michaelis constant of PEPA increases with an increase in pH, whereas the Michaelis constants of K^+ and Mg^{++} decrease with an increase in pH.

Although no immediate parallel can be drawn at present between cation-PPFase interactions and active cation transport, study of such interactions may give some hint as to the nature of the cation-transporting groups in the cell surface, and how the cations interact with these groups. These findings and others of a similar nature may eventually be of use not only in the understanding of the basis for cation transport, but also the basis of physiological antagonisms and the mechanism of enzyme action.

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A KINETIC ANALYSIS OF CATION INTERACTIONS IN THE PYRUVIC PHOSPHOFERASE SYSTEM OF RABBIT ERYTHROCYTES

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INTRODUCTION

During the last 25 years attention has been directed toward the composition of the intracellular fluid, and of late, toward how this environment is maintained. The phenomenon of active cation transport through living cell membranes is fundamental to the understanding of many of the problems encountered today in the understanding of cellular physiology. Although the high K^+-content of the intracellular fluid environment has long been recognized, knowledge of its influence on metabolic activities is only of recent origin.

The red blood cell has long been a subject of study by the biochemist since, in contradistinction to somatic cells, it is easily obtainable, easily isolated, and may be suspended in media other than plasma. The properties, structure and function of the plasma membrane of the erythrocyte are especially amenable to study.

Transfusion therapy and the development of blood banks have given impetus to the search for mechanisms responsible for the control of cation gradients in the erythrocyte. Interest has arisen in such gradients, since besides the decreased ability to metabolize glucose, increased osmotic and mechanical fragility, there is also a loss of potassium from within the cell with replacement by sodium (1), during the *in vitro* storage phase at 4^oC. in the acid-citrate-dextrose medium. There is also decreased ability of the cells to survive when transfused into a recipient (2, 3). Customary demands of blood banks are increased when there are superimposed the needs of war areas, and it is without doubt that storage for longer periods of time than the present permissible limit of 21 days (2, 3) would be highly desirable.

The erythrocyte membrane being semipermeable has, of course, come under careful scrutiny as the possible regulator of cation transport. Denstedt (4) has recognized that the innovation of flame photometers, radioactive isotopes, and the tremendous advances in the fields of general metabolism and enzymology, have led to a change in the concept of the semipermeable membrane of the erythrocyte in recent years. The change has been from a search for an alteration in structure on storage (the physical approach), to a realization that the property of semipermeability is a manifestation of the dynamic state of the cell. A cell's permeability cannot be regarded as being governed largely by its chemical and physical properties. Thus any condition which interferes with the metabolism of the cell will also influence the permeability of the membrane; this is the biochemical approach.

The physical approach to a study of erythrocyte permeability is still used to advantage in determining the structure of the membrane. Hillier and Hoffman (5) using electron microscope data of human erythrocyte ghosts, estimate the thickness of the plasma membrane to be 60 to 100 angstroms. Their data suggest that the erythrocyte membrane consists of a mosaic of glycolipoprotein plaques, 30 angstroms in height and 200 angstroms in diameter. They believe these plaques are situated on the outside of a fibrous network, which forms the inner lining of the plasma membrane. The plaques can be removed by ether, suggesting that the plaques are held to the fibrous component by lipids. The minimal "pore size" between plaques has been calculated to be ca. 35 angstroms, and the "pores" are likely in the nature of aqueous channels.

Ponder (6) considers the cell and ghost to contain at least four proteins. Moskowitz et al. (7) isolated a lipoprotein from ghosts, which

they have called "elinin." They found all of the activity of the Rh, A, and B factors to reside with this elinin fraction. It has been suggested (5) that the plaques are identical with this elinin fraction.

Increased permeability to both electrolytes and nonelectrolytes by the action of lipase (8), n-butyl alcohol (9), trypsin (10) and x-radiation (11) may be attributed to a change in molecular structure of the plasma membrane by these agents.

Attempts to duplicate the ion selective properties of physiological membranes date prior to 1930. The preparation of various types of membranes which exhibit selectivity and, or, specificity has been reviewed by Gregor et al. (12). These authors have recently prepared a polysterene-sulfonic acid-dynel membrane, for example, which had only a small anion leak, and was highly specific toward calcium ions.

West (13) notes that tissues, like enzymes, are largely colloidal and are interspersed with a network of membranes possessing enormous surface areas. Adsorption processes taking place at these membranes may promote metal chemical reactions, and may cause changes in surface tension and cell consistency. West further attributes the high lipid content of cell membranes, and resultant high permeability, to fat-soluble substances, to the fact that substances which lower surface tension tend to concentrate at the surface.

Most work has been carried out on species which have erythrocytes of the high potassium, low sodium type. Keitel et al. (14) have compared the electrolyte composition of plasma and erythrocytes of humans. The mean sodium concentration in plasma and erythrocytes was 142.6 mM. and 43.2 mM per Kg. of packed cells respectively; that of potassium in plasma and erythrocytes was 4.04 mM. and 267.9 mM. per Kg. of packed cells. Erythrocytes of various

species show a wide variation in the ratio of sodium to potassium (14); i.e. 1 sodium : 9 potassium in the pig and 17 sodium : 1 K in the cat, although the sodium ion concentration in the plasma is always larger than the potassium ion concentration. Unless stated to the contrary, all reference in this thesis will be directed to the high potassium, low sodium type of mammalian erythrocyte.

The concept that semipermeability of the membrane is not a fixed property but a manifestation of the metabolic state of the cell gains support from Altman's observation (15) that there is a rapid turnover of C-14 constituents in the stroma.

The ability of the erythrocyte to maintain a high level of internal potassium and low level of internal sodium is believed to be linked with the metabolic activity of the cell (16). Such active transport represents work, and therefore requires energy. Any condition interfering with metabolism should thus influence the permeability of the erythrocyte toward these two ions.

The effect of such variables as pH and addition of dextrose to the suspending medium on erythrocyte metabolism and permeability has been investigated by Rapoport (17), Parpart (18), Osborne (19) and Green (20). The reversible exchange of sodium and potassium on storage at $4^{\circ}C$. has been reported by Harris (21) and Maizels (22). They found the loss of potassium and gain of sodium by erythrocytes are reversed by incubating the cells at a higher temperature in the presence of glucose. Maizels and Whittaker also observed this reconstitution of electrolyte composition, when the cells are placed back in circulation (23).

A number of reviews dealing with sodium and potassium transport across the red cell membrane are available (24, 25, 26, 27, 28). A review

of experimental data has led Tosteson (28) to conclude that potassium efflux and sodium influx are almost entirely by slow diffusion whereas potassium influx and sodium efflux are mediated almost entirely by chemical reactions which are linked to glycolysis by some unknown intermediates. High potassium,low sodium erythrocytes must maintain their ionic composition by pumping potassium in and sodium out. Some of the more important theories advanced to explain the pumping action will be briefly reviewed.

Lundegardh (29) proposed one of the first theories, in which he suggested the surface of the cell was made up of a mosaic of strongly acidic and weakly basic groups; the former preponderate and give the surface a net negative charge. He suggested the acid groups and basic groups could act as cation and anion carriers respectively. These charged groups were thought of as being built into the surface of the red blood cell, and of being capable of acting as "swinging doors", i.e., swinging around to exchange attached ions with the interior of the cell.

Osterhout (30) has concluded that active transport occurs only with cations. His model for active transport assumes a carrier molecule exists on the cell surface which forms a lipid-soluble complex with the cation, the cation carrier complex dissociating on the inner surface, after traversing the membrane. Osterhout and Stanley (31) have devised a homogenous membrane having the properties of a living membrane in so far as it is able to accumulate potassium ions.

Since 1949 Greig (32a-h) has been attempting to link cation transport in the erythrocyte with the choline esterase-choline acetylase system. She found that suspending dog erythrocytes in $NaHCO_3$ caused an exchange of sodium for potassium within the cell. Adding acetyl choline and maintaining acetyl choline esterase activity decreased these changes. Acetyl choline esterase

inhibitors such as physostigmine, methylene blue, and methadon block replacement of K in cells on addition of acetyl choline. She has carried out similar experiments with human cells (32h). Numerous objections have been advanced, one of the most important being evidence against the ability of the erythrocyte to synthesize acetyl choline presented by Mathias and Sheppard (33), and Collier and Solvonuk (34). Solomon (35) proposed a 3-compartment system for sodium and potassium transport. Since he regards the plasma as one compartment the red blood cell is considered to have two functional compartments, having the property of slow and fast cation exchange. More recently Solomon et al. (36) have found evidence for possible carrier molecules responsible for the transport of cations across cell membranes. The material extracted from dried blood had properties considered requisite of such carriers. It bound potassium preferentially to sodium, was soluble in lipid solvents, and dissociated in water.

Glynn (37) using radioactive tracers to study the movement of sodium and potassium in human red blood cells, established that, for active flux, which appears to be geared to glycolysis, there is a 1:1 ratio in the rate of exchange of potassium for sodium in the erythrocyte. Passive fluxes can be described by passive diffusion. They are predominantly outward for potassium and inward for sodium, the rate being proportional to the gradient. Sodium efflux appears to consist of two components; an active component tightly linked to potassium influx and a passive component not linked to potassium influx, nor glycolysis.

The problem of cation transport is of course not peculiar only to erythrocytes. A normal sodium to potassium gradient is maintained in muscle cells. A link between active sodium effusion and potassium influx has been demonstrated in frog muscle and cephalopod nerves (38, 39). Further, sodium

appears to be more concentrated in the nuclei than in the cytoplasm at large (40). Recently (41) simultaneous measurements of active ion transport and oxygen consumption of short circuited frog skin has been used to test the "Redox" hypothesis of active ion transport. Scott et al. (42) note that the marine alga, Ulva lactuca, although living in a medium of high sodium and low potassium, as do most cells in higher organisms, exhibits the reverse state of affairs in its cell. In addition most plants (43), and also the nervous tissue of animals (44) exhibit this same gradient within their tissues. Active excretion by the kidney tubules (45) and acid secretion (46) are of no doubt related to active transport.

That the discrimination between sodium and potassium may operate at the enzyme level has been forwarded by several investigators (47, 48, 49, 28, 37) and serves as a basis for the "enzyme hypothesis" for the control of active Na^+ and K^+ transport. As suggested by Glynn (37) it is not necessary that the enzyme have different affinities for these two ions but merely that it should be more active when binding one than the other.

Although a number of enzymes are activated or inhibited by univalent ions (37, 50), in particular by sodium and potassium, most attention has been focussed on pyruvic phosphoferase as a possible regulator of the cation gradient in the red blood cell. Lehninger has pointed out (48) that it would be more desirable to have more data on the metal interactions of this enzyme, since it requires a univalent and a divalent ion for activation.

A study of cation interactions, both with activators and inhibitors, and an attempt to interpret the results of the cation antagonisms found, has already begun (47, 49, 51). It is a continuation of the kinetic analysis of cation interactions in the pyruvic phosphoferase system, and an attempt to elucidate their mechanism, with which this thesis is mainly concerned.

Phosphoenol pyruvic acid, the substrate for this enzyme, was discovered by Lohmannand Meyerhof in 1934 (52). They showed it to be an intermediate in the transfer of phosphate from 2-phospho glyceric acid to the adenylic system, a reaction discovered by Parnas et al. (53). Further research showed that two metal ions were required for activity. Lohmann and Meyerhof (52) demonstrated that magnesium ion was required; activation by potassium was demonstrated by Boyer, Lardy and Phillips (54). Kachmar and Boyer (47, 62) have made a kinetic study of this enzyme in which they showed the indispensibility of potassium and magnesium ions, and the inhibition by sodium, lithium and calcium. Other ions can substitute (47, 51); potassium can be replaced by ammonium or rubidium ions, and cobalt and manganese can replace magnesium ions to some degree.

Meyerhof et al. (55) using the dialyzed muscle enzyme obtained results which suggested the catalyzed reaction was nonreversible. However, Lardy and Zeigler (63) were able to demonstrate the incorporation of P^{32} into phosphoenol pyruvate using the same conditions as the former investigators, but with the addition of potassium to the dialyzed enzyme. It is interesting to note that although potassium appears to be necessary for both the forward and reverse reactions with the enzyme from animal tissues, it is not required by the plant enzyme (57, 58). Meyerhof and Oesper (59) have determined the equilibrium constant to be 2000, at 30°C. in bicarbonate buffer. They have also determined the quotient for the forward and reverse reactions to be 500.

Boyer (60) has found a strong activation by potassium ion of this enzyme obtained from various tissues of eleven vertebrate and invertebrate species examined. Solvonuk and Collier (63) have determined the pyruvic phosphoferase activity in the erythrocytes of six mammalian species and found

erythrocytes of low potassium high sodium type tend to have lower pyruvic phosphoferase activity. Their reported inhibition by iodoacetate (63) has been suggested by Tosteson (28) as a possible mechanism of its action in reducing potassium influx and blocking of lactate formation in duck erythrocytes.

Pyruvic phosphoferase plays an all important role in the cellular glycolytic mechanism. It catalyzes the following reaction:

$$* \text{ PEPA} + \text{ADP} \xrightleftharpoons{Mg^{++}\ K^{+}} \text{pyruveate} + \text{ATP}$$

Because of the nature of the activation and inhibition by cations of this enzyme system, some interpretation of the nature of important physiological cation antagonisms may be gained from its study.

Both in vivo and in vitro studies of ion antagonisms in biological systems have been extensively studied and recently reviewed (64, 65, 66, 48). Original studies began with Ringer's investigations (67) on the toxic effects of sodium chloride on the irritability of muscle. The importance of the Mg-Ca antagonism in muscle has been recognized (68, 69). It is of interest to note that Steinberg (70) has constructed a biological periodic table and has attempted to explain their physiological effects on the basis of physical correlations.

Any attempt to review, in an introduction, the large amount of data now available on cation antagonisms in biological systems, both unicellular and multicellular, plant and animal, would be impossible. However, it may be stated that the antagonistic effects observed during in vivo and in vitro studies, in a number of cases, are without doubt manifestations of physiological control over the activity of metal requiring enzymes.

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*The following abbreviations will be used:

PEPA = phospho(enol) pyruvate
ADP = adenosine diphosphate
ATP = adenosine triphosphate
PPFase = pyruvic phosphoferase
Tris = tris(hydroxymethyl) aminomethane hydrochloride

II. EXPERIMENTAL

A. EXPERIMENTAL PROCEDURE

1. Introduction

The source of PPFase (pyruvic phosphoferase) has been exclusively the dialyzed hemolysate of rabbit erythrocytes. Solvonuk and Collier (51) have established this enzyme to be located in the soluble interior of the erythrocyte, the 'ghosts' being devoid of activity. Dialysis in the presence of 2% albumin was used to remove endogenous substrates and ions. Albumin was required to prevent inactivation of PPFase on dialysis.

Reports of an active DPNase (71) and possibly an active ATPase (72) in the erythrocyte membrane suggest that the removal of the ghosts is desirable. It is also of importance to note that Gabrio et al. (73) found ATPase to be localized largely in the soluble fraction of the cell, and to be activated by magnesium and inhibited by calcium ions. They consider it to be in a latent state in the intact cell but to become more active as the cell loses its integrity. The presence of such an ATPase in the hemolysate would undoubtedly interfere with any experimental studies in which ATP must be added to the assay. Although no other such interfering enzymes are known to exist in the erythrocyte, it is interesting to note that a magnesium ion-requiring phosphomonoesterase capable of hydrolyzing PEPA (phospho(enol) pyruvate) has been demonstrated in milk (75).

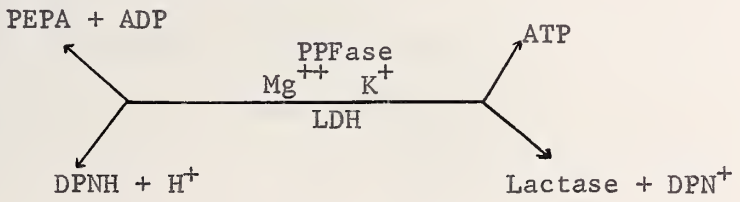
Fortunately, there appears to be no appreciable binding of sodium and potassium by hemoglobin (76) although three plasma fractions showed a significant binding of both at pH 7.5.

Instead of the colorimetric method for free pyruvate upon which the previous assay for PPFase was based (47, 51) the present investigation employed

the spectrophotometric method of Negelein (77), as described by Bucher and Pfleiderer (78a). In this method the PPFase system is coupled with LDH (lactic dehydrogenase) in the presence of DPNH (reduced diphosphopyridine nucleotide). The pyruvate, produced from the transfer of "high energy" phosphate from PEPA to ADP, is reduced to lactate, and the oxidation of the reduced coenzyme (DPNH), which has an absorption maximum at 340 mμ, is followed spectrophotometrically. However, since so little light was transmitted at 340 mμ due to high absorption by hemoglobin, 320 mμ was selected as a compromise between the region of least interference by hemoglobin and the region of maximum absorption by DPNH. It is of interest to note that decomposition of pyridine nucleotides occurs only when they are exposed to high intensity U.V. in the region of 260 (79).

The activity measurements were carried out using a modified Beckman DU Spectrophotometer, with photovolt photomultiplier photometer. The temperature of the cell compartment was held at 37°C. by circulating water from a thermostatically controlled water reservoir through a thermospacer arrangement.

The compound optical assay method described for the measurement of PPFase activity may be summarized as:



The coupling enzyme LDH must be present in excess in order that the rate of oxidation of DPNH is not the rate-limiting step. Ensuring an excess by direct addition of crystalline LDH prepared from muscle (Worthington Biochem. Corp.) was unsuccessful since it was found to contain a high PPFase activity. It is widely accepted that PPFase contaminates many LDH preparations (78c). However, electrophoretically resolved component A (78d) is free of PPFase activity.

We first assumed the LDH to be present in excess, since the velocity of the measured reaction appeared to be independent of the concentration of DPNH. Later we were better able to test this assumption through the kindness of Dr. J. B. Neilands of the University of California, who supplied us with an electrophoretically resolved LDH preparation. Further, as a coupling enzyme, it is a notable property of LDH that its activity is unaffected by any of the cations used in the present investigation (78d, 80).

Other spectrophotometric methods for measuring PPFase activity are available. Measurement of U.V. absorption of PEPA at 240 mμ (78b) would be ideal for studying both reverse and forward reactions. However, absorption by other components of hemolysate, even after removal of hemoglobin using the water-ethanol-chloroform mixture, is much too great at 240mμ .

The substrate, PEPA was originally obtained as the silver barium salt (Calif. Found. for Biochem. Research) from which the potassium salt was prepared (47). Enzyme inhibition was sometimes encountered and some of the results were atypical, possibly as a result of incomplete removal of silver ions. The conversion to production of the tricyclohexyl amine salt (Calif. Found. for Biochem. Research) is regarded as a major improvement.

Since distilled water from the reflux still used in the Department of Biochemistry at the present time has been shown to contain 0.3 to 0.4 p.p.m. of available chlorine which might interfere with enzyme activity to a significant extent (81), all distilled water has been made chlorine free by passage through an ion-exchange type water purifier.

Due to the nature of the assay, a maximum of two determinations can be run simultaneously in the spectrophotometer. Thus most studies, requiring a number of determinations of enzyme activity, will necessitate that deterioration of reagents be kept to a minimum. The substrate, hemolysate, and coenzyme

preparations have been stored in stoppered test tubes at ice bath temperature over the extended periods of time required.

Although more time consuming, the distinct advantage of the spectrophotometric method over the colorimetric method lies in the fact that any quantitative treatment of reaction kinetics should use the initial portion of the time vs. activity curve. By basing activity measurements on the initial velocities it has been possible to employ lower concentrations of coenzyme (ADP), possibly an important consideration, when the extent of ion binding by this coenzyme is to be neglected.

2. Preparation of Hemolysate Containing PPFase and LDH

Ten ml. of rabbit blodd, obtained by heart puncture, was collected in heparanized test tubes. A portion of the blood was centrifuged and the plasma was removed by aspiration. The erythrocytes were then washed twice at the centrifuge with isotonic KCl (0.154 M), the supernatant liquid and buffy layer also being removed by aspiration. The packed cells (Ca 2 ml.) were hemolyzed by the addition of cool distilled water and were diluted to approximately 8 millilitres. The hemolysate was dialyzed against a large volume of distilled water at 5°C. for 15 hours. Removal of the ghosts was facilitated by adjusting the pH of the hemolysate to approximately 6.9 before centrifuging, then readjusting to 7.4 after removal of the 'ghosts'. The 'ghosts' were removed by centrifuging at 11,000 r.p.m. for 15 minutes using a Model PR-1 International Refrigerated Centrifuge with a multispeed attachment. The ghost-free hemolysate was then diluted to give a 1:20 dilution based on the original packed erythrocyte volume.

The criterion for the final dilution of the hemolysate for any given series of determinations has been considered that which will give an absorbancy

in the vicinity of 0.1 O.D. units* in 3 minutes in that single determination in such a series which most closely approximates the conditions considered optimal for activity of the enzyme. For velocities much greater than 0.1 O.D. units per 3 minutes small changes in velocity are less easily discernible. On the other hand, using dilutions giving maximum velocities much lower than 0.1 O.D. units per 3 minutes will limit the range of enzyme activities which can be measured accurately, since velocities in any series located at the lower limits are also subject to errors of measurement. These errors may be attributed to deterioration of the enzyme and coenzymes, and instability of the instrument over the relatively long periods of time necessary to obtain a measurable change in absorbancy.

The enzyme activity deteriorates on standing even at ice bath temperature and it is necessary to recheck the activity at frequent intervals of time under standard conditions, during any series of extended measurements.

3. Preparation of Reagents

Many of the reagents are somewhat labile and are subject to contamination by microorganisms. Consequently the amounts prepared at any one time are a compromise between practicality and rate of deterioration.

The concentrations of the KCl and $MgSO_4$ solutions used were 1.0 M and 0.05 M respectively, and those of the NaCl and $CaCl_2$ solutions, 1.0 M and 0.035 M.

Tris, i.e. tris(hydroxymethyl) aminomethane hydrochloride buffers whose buffering actions lie in the physiological pH range 7-9 were prepared as follows: 4.845 gm. of tris (Sigma Chem. Co.) was dissolved in water and adjusted to the proper pH with HCl, and to a final volume of 100 ml. with distilled water. Since tris buffers have an appreciable temperature coefficient,

*O.D. = Optical density

they have been prepared at 23° as described, the appropriate correction for the lower pH at 37° being obtained from a table prepared by Gomori (82). The corrections, obtained by potentiometric measurement of tris buffers at 23°C. and 37°C., range from 0.13 to 0.15 pH units.

Since the buffering capacity of tris buffer has a limited range, trismaleate buffer (83) for use in the pH range below 7.0 has been prepared from a stock solution, containing 24 gm. of tris and 19.6 gm. of maleic anhydride (Eastman Kodak Co.) per 100 millilitres. Ten ml. of this stock solution was adjusted to the desired pH with base and to a total volume of 50 millilitres. A 10% solution of tetramethyl ammonium hydroxide (Eastman Kodak Co.) was used as base rather than KOH or NaOH since the PPFase activity appears to be uninfluenced by the tetramethyl ammonium ion (P. 76).

These buffers were refrigerated when not in use and were freshly prepared twice a week since they easily become contaminated with microorganisms.

A 0.01 M solution of PEPA was prepared from the silver barium salt (Calif. Found. Biochem. Research) by tituration with HCl and K_2SO_4. To 45 mg. of PEPA in a test tube in an ice bath, 2.0 ml. of 0.1 N HCl were added with stirring. Subsequently 0.4 ml. of 0.5 M K_2SO_4 was added followed by 4 ml. of water. After digesting for 1 hour at 37°C. the precipitate was centrifuged down. Further additions of K_2SO_4 were made, followed by 30 min. digestion periods and centrifugation until a bare excess of K_2SO_4 was present. After separation from the precipitate the pH of the solution was adjusted to 7.4 with KOH, and the final volume was 10 millilitres. The preparation was stored frozen, and any $BaSO_4$ remaining after thawing was removed by centrifugation.

To prepare a 0.01 M solution of PEPA from the tricyclohexyl amine salt, 46.6 mg. was dissolved in water, and the pH was adjusted to 7.5 with HCl; the final volume was 10 millilitres. The substrate solution was stored frozen.

Two ml. of either 0.02, 0.04 or 0.06 M solutions of ADP (Sigma) were prepared depending upon the magnitude of the final concentration of ADP required in the reaction mixture. Since ADP is added to the reaction mixture just prior to taking velocity readings, it is necessary that the volume of solution added be of the order of 0.1 ml. since larger volumes cause a noticeable cooling effect while smaller volumes increase the pipetting error. The pH was adjusted to 7.0 with KOH since ADP is labile in both acidic and basic solutions. Deterioration may become significant after 4 to 5 hours.

A 0.0015 M solution of DPNH was prepared by dissolving 5 mg. DPNH (Sigma) in 5 ml. of water. Slight deterioration is not critical. Fresh solutions were prepared daily.

4. Assay Procedure

A typical incubation mixture consisted of the following components, final molar concentrations being indicated: $MgSO_4$, 0.008; KCl, 0.070; tris, 0.050, pH 7.5; PEPA, 0.00078; DPNH, 0.00013; and dialyzed hemolysate of the appropriate dilution. This mixture was made up to a total volume of 3.00 - x ml., where x represents the volume of ADP which is added last to start the reaction, in a 1" cm. quartz cell. The contents of the quartz cell (fitted with a stopper) were mixed by inverting the cell 5 to 6 times. The cell was then placed in a constant temperature water bath at 37°C. for 3 minutes for thermoequilibrium. The cell was quickly dried externally and was transferred to the cell compartment, also at 37°C. for another 3 minutes' incubation. During the incubation periods, any free pyruvate present in the PEPA preparation was reduced; the concentrations of free pyruvate were, however, very slight.

After a total of 6 minutes' incubation (viz. in the water bath and cell compartment), x ml. of ADP was pipetted into the reaction mixture to

give a final volume of 3.000 ml. Mixing was quickly accomplished by inverting the cell holder with cell several times. The cover to the cell compartment was kept in place as much of the time as possible between transfers.

Immediately after starting the reaction transmittancy readings were taken from the galvanometer of the photovolt multiplier photometer at 30 second intervals over a period of 6 minutes, or longer, if the activity was very low. The final molarity of DPNH of 0.00013 is usually sufficient to provide for an absorbancy change of 0.30 O.D. units. In order to attain most accurate results the slit width on the Beckman spectrophotometer was adjusted so that the initial readings were not greater than 0.3 O.D. units in magnitude at 320mμ.

In most cases the procedure involved running two assays at once, readings being taken on each individual assay alternately, every 15 seconds.

Since the stability of the photovolt multiplier photometer was somewhat unreliable its stability was checked against a standard, after every second reading was taken. The only qualification of the standard in the optical assay method is that it should not change optical density. The density of the artificial standard employed, consisting of a pyrex cuvette, a silica cell spacer and strips of cellophane, was adjusted so as to give a transmittancy of 100% when the slit width of the spectrophotometer was adjusted to give an initial optical density of approximately 0.3 with respect to the assay mixture.

When the inhibiting ions Na^+ and Ca^{++} were to be used as part of the assay mixture in inhibition studies of the PPFase system, the procedure was essentially the same, except for the incubation step. For inhibition studies, appropriate volumes of enzyme, substrate and inhibiting ion were added to the cuvette and were made up to a volume of 2.00 ml. After incubating at 37°C.

for 20 minutes, DPNH, tris buffer and water were then added, making a total volume of 3-x ml. After a further 6 minutes of incubation the reaction was started by adding x ml. of ADP. The 20 minute incubation period was found to be necessary in order to obtain reproducible results with both inhibiting ions. Tris buffer and DPNH were omitted from the assay during the first incubation period since the former seemed to have an adverse effect on the stability of the enzyme, especially at pH values removed from the optimum, and the latter showed some deterioration during the relatively long incubation period.

A control was run with each inhibition assay in order to obtain most accurate results. For the control an equivalent amount of water was substituted for the inhibitor prior to the 20 minute incubation period. The final volume has been 3.0 ml. in all determinations.

LDH activity has been measured by omitting ADP and substituting an appropriate amount of pyruvate for PEPA in the PPFase assay mixture. In addition to the necessary components, viz. LDH, DPNH and pyruvate, the activating ions were also included.

5. Unit of PPFase Activity

The transmittancy readings obtained from the galvanometer at 30 second intervals were converted to O.D. units. The unit employed is defined in the same way as that used for α-glycerophosphate dehydrogenase (78e) except for the substitution of minutes for seconds.

From a plot of O.D. vs. time in minutes (Fig. 1), the time required for an O.D. change of 0.1 was determined from the graph. A unit is defined as follows:

$$U = \frac{100}{\text{minutes for a change of O.D. of 0.1}}$$

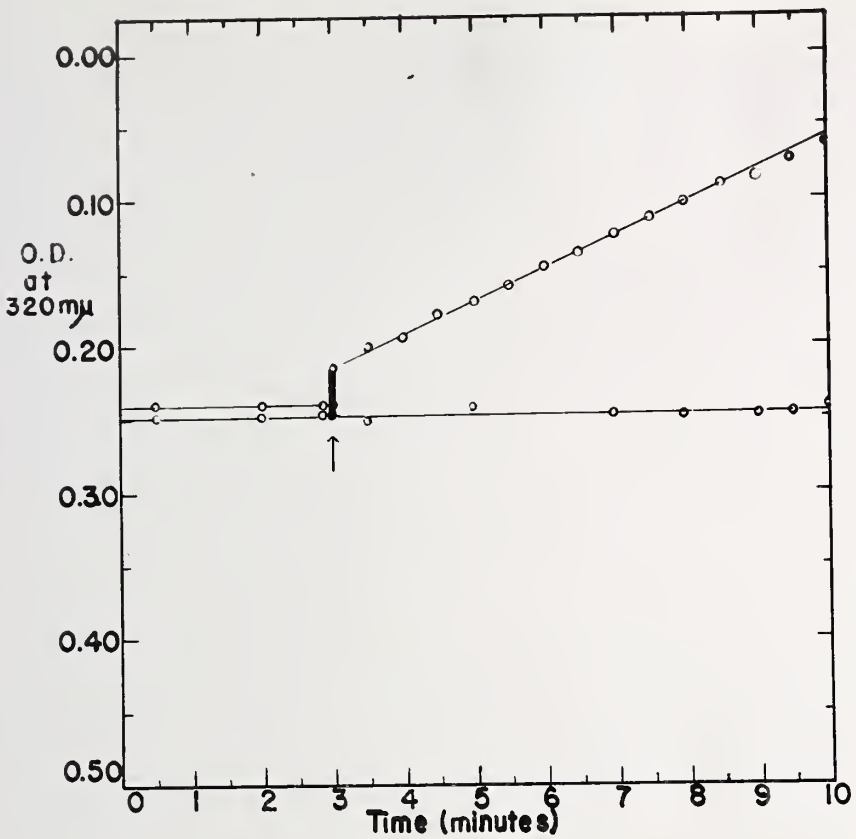


Fig. 1
Measurement of PPFase Activity Using
the Optical Assay Method

At zero time both assays consist of the following final molar concentrations: 0.008 M $MgSO_4$, 0.075 M KCl, and 0.05 M tris buffer, hemolysate 0.00078 M PEPA, and 0.00013 M DPNH. At three minutes, as indicated by the arrow, 0.0005 M ADP has been added to one of the assays. No change in O.D. occurs during the entire 10-minute period in that assay to which no ADP was added.

For comparative purposes only the same unit has been adopted for expressing LDH activity.

At zero time both assays consist of the following final molar concentrations: 0.008 M $MgSO_4$, 0.075 M KCl, 0.05 M tris buffer, hemolysate 0.00078 M PEPA, and 0.00013 M DPNH. At three minutes, as indicated by the arrow, 0.0005 M ADP has been added to one of the assays. No change in O.D. occurs during the entire 10 minute period in that assay to which no ADP was added.

6. Preliminary Studies on the Coupled PPFase-LDH System

(i) Selection of Wavelength

TABLE I
Selection of Wavelength for the Optical Assay

Wavelength	DPNH only (O.D. units)	Assay Mixture less DPNH (O.D. units)
315	0.549	0.089
*320	0.594	0.218
325	0.643	0.380
330	0.678	0.538
335	0.694	0.663
*340	0.699	0.770
345	0.678	0.912
350	0.638	1.046

Absorbancy changes have been measured at 320 mμ rather than at 340 mμ where absorption by other components of the system is much less (Table I). Using 320 mμ it was possible to use quite high hemolysate concentrations, yet take initial readings around 0.3 O.D. units; i.e. where the change in transmittancy per change in O.D. is quite large, thus reducing the experimental error. This was possible at 340 mμ only using low hemolysate concentrations.

(ii) Binding of Sodium and Potassium by Hemoglobin and Plasma Proteins

Equilibrium dialysis has been used to demonstrate the extent of

binding of Na^+ and K^+ by hemoglobin and plasma proteins. Sodium and potassium determinations were carried out on a Beckman DU spectrophotometer equipped with a photo multiplier attachment and an acetylene burner No. 4030. The instrument was standardized against values obtained previously by Solvonuk (85) which had been used in preparing standard curves for both serum and erythrocyte sodium and potassium determinations. Since the extent of binding by plasma proteins is so great that transmittancy readings obtained go far beyond the data presently available on standard curves, transmittancy readings are presented rather than concentrations (Table 2).

For binding studies with hemoglobin the red blood cells were diluted 1:200 and for binding studies with plasma, it was diluted 1:50. Background readings have been subtracted from transmittancy readings.

TABLE 2
Binding of Na^+ and K^+ by Hemoglobin and Plasma Proteins of Rabbit Blood at pH 7.4

Fraction	Cation	Dialysate (% T)	Fraction being dialyzed (% T)
Hemoglobin	K^+	31	31
	Na^+	9	9
Plasma	K^+	15	57
	Na^+	22	78

(iii) Dialysis of the Hemolysate

(a) Effect of Dialysis on Enzyme Activity

Neglecting to add albumin to the hemolysate prior to dialysis on several occasions did not result in any remarkable loss in activity. To test the effect of addition of 2% albumin hemolysate obtained from rabbit erythrocytes (1:3 dilution) was divided into parts; to one part no albumin was added, while to the other part 2% bovine serum albumin (Armour Laboratories Ltd.) was added. Both were dialyzed for 14 hours against distilled water at

5°C. Prior to centrifuging the dialyzed hemolysates, 2% albumin was added to the hemolysate to which no albumin was originally added. The 'ghosts' and most of the albumin were centrifuged out at 12,000 r.p.m. The O.D. of 1:25 dilutions of both hemolysates were examined at 320mμ. The hemolysate to which no albumin was added prior to dialysis was slightly more concentrated and was diluted down until both hemolysates had the same absorbancy.

The results of PPFase activity measurements on both hemolysates are shown in Table 3(a).

TABLE 3(a)
Effect of Adding 2% Albumin to Hemolysate of Rabbit R.B.C.s prior to Dialysis, on PPFase Activity

Additions Prior to Dialysis	Initial PPFase Activity*	PPFase Activity after Dilution
Hemolysate only	36.3	28.0
Hemolysate + 2% Albumin	28.6	28.6

*Before diluting both to the same O.D.

(b) Removal of Endogenous ADP and Other Factors by Dialysis

Removal of endogenous substrates, coenzymes and metal ions is extremely important. Removal of ADP and PEPA in particular obviates the necessity of running blanks.

TABLE 3(b)
Removal of Endogenous ADP from Hemolysate by Dialysis

Description	Undialyzed Hemolysate	Dialyzed Hemolysate
	(units PPFase activity)	
Complete assay mixture (+ADP)	67	42
Assay mixture without addition of ADP	22	0

(iv) Effect of Cyclohexylamine on the Enzyme

Since the tricyclohexylamine salt of PEPA was used, it was important to demonstrate that this base did not have any effect on the PPFase system at the concentrations used. The effect of adding cyclohexylamine at concentrations of activating ions considered to be suboptimum has also been tested to determine if any complex formation occurs. The cyclohexylamine (Eastman Kodak) was adjusted to pH 7.5 before use.

TABLE 4
Effect of Cyclohexylamine on the PPFase System

Molarity of Cyclohexylamine	Conc. of Mg^{++} and K^{+}	% Inhibition
0.0125	Optimum	12.7
0.005	Optimum	7.0
0.003	Optimum	0
0.003	Suboptimum	0

In view of the fact concentrations of PEPA in excess of 0.0012 M (equivalent to 0.0036 M of cyclohexylamine) were rarely employed, removal of the base was unnecessary. There is no evidence of binding of cations by 0.003 M cyclohexylamine.

(v) Effect of Enzyme Concentration

(a) PPFase

TABLE 5
Effect of Hemolysate Concentration
on Measured PPFase Activity

Relative enzyme conc.	Activity (units)
0.5	9.4
1.0	18.9
2.0	37.1
2.5	47.6

(b) LDH

The lactic dehydrogenase activity of erythrocytes is rather high.

Replacing PEPA by the same molarity of pyruvate, and defining the LDH unit in the same manner as the PPFase unit, for comparative purposes only, gave LDH activity of around 3,000 units in assays which show less than 40 units of PPFase activity.

Lactic dehydrogenase electrophoretically enriched in component A* shows no PPFase activity whereas unresolved LDH shows considerable PPFase activity. Addition of component A to PPFase assays in the velocity ranges normally employed resulted in an increase in velocity of from 2 to 5%. At velocities much higher than those used this error was increased to 8%. In an experiment on calcium inhibition of PPFase, 0.003 M Ca^{++} produced 55.5% inhibition in assays to which no LDH was added and 58% inhibition in assays to which the LDH preparation was added.

(vi) Optimal Requirements

(a) Optimum K^+ Concentration

TABLE 6
Optimal K^+ Requirements of the PPFase System

K^+ Conc. (final) M	Activity (units)
nil	0.000
0.01	7.5
0.06	16.1
0.075	16.9
0.09	16.3
0.25	12.0

The optimum was estimated to be 0.075 M.

*We are indebted to Dr. J. B. Neilands, University of California, Berkeley, for a gift of LDH, electrophoretically enriched in component A.

(b) Optimum Mg^{++} Concentration

TABLE 7
Optimal Mg^{++} Requirements of the PPFase System

Mg^{++} Conc. (final) M	Activity (units)
nil	0.000
0.0001	3.39
0.001	20.8
0.006	32.8
0.008	33.9
0.016	25.2

The optimum has been considered to be 0.008 M.

(c) Optimum PEPA Concentration

TABLE 8 (a)
Optimal PEPA Requirements of the PPFase System

PEPA Conc. (final) M	Activity (units)
0.0001	10.3
0.0005	30.3
0.0007	32.8
0.00078	34.5
0.0020	34.5

0.00078 M PEPA has been used as the optimum.

TABLE 8 (b)
Apparent Change of Optimum PEPA Concentration* with pH

pH	Optimum PEPA Conc. (final) M
6.8	0.00065
7.5	0.00078
8.0	0.00085

*The optimums have been estimated from plots of activity vs. PEPA conc. at the various pH values.

From the Lineweaver Burk. plot, Fig. 2, the Km has been estimated to be 4×10^{-4} M using 5×10^{-4} M ADP.

(d) Effect of ADP Concentration

TABLE 9
Effect of ADP Concentration on PPFase Activity

ADP Conc. (final) M	Activity (units)
0.00013	8.1
0.00026	11.1
0.00052	15.6
0.00078	17.2
0.00104	18.9
0.00156	21.8
0.00208	25.0
0.00234	26.3
0.00260	27.8
0.00286	28.6
0.00325	28.6
0.00650	29.4

The graph of PPFase activity vs. ADP concentration (Fig. 3) shows that between the ADP concentrations of 0.00023 M and approximately 0.0027 M the maximum initial velocity is proportional to the concentration of ADP. The slope of the line in the region where the initial velocity is proportional to the ADP concentration, appears to be proportional to the enzyme concentration. Determinations were run at ADP concentrations of 0.0004 M, 0.0006 M, 0.0008 M and 0.0010 M using relative enzyme concentrations of 1.0, 0.50 and 0.75. The ratio of the relative enzyme concentration to the slope of the line was,

$$1.0 : 0.75 : 0.50 = 1.0 : 0.70 : 0.48$$

(If additions of ADP greater than 0.1 ml. were required, the ADP was warmed to 37°C. before addition.)

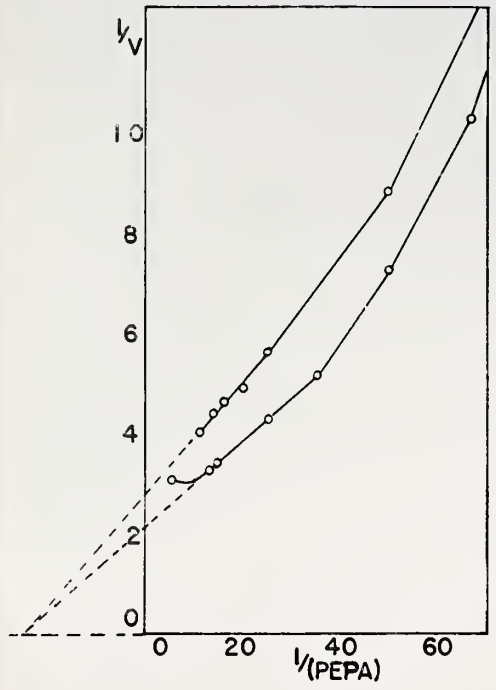


Fig. 2. A Lineweaver-Burk Plot showing the effect of PEPA concentration on PPFase Activity (pH 7.5). The ADP concentration was 0.0005 M. Plots at two different enzyme concentrations are shown.

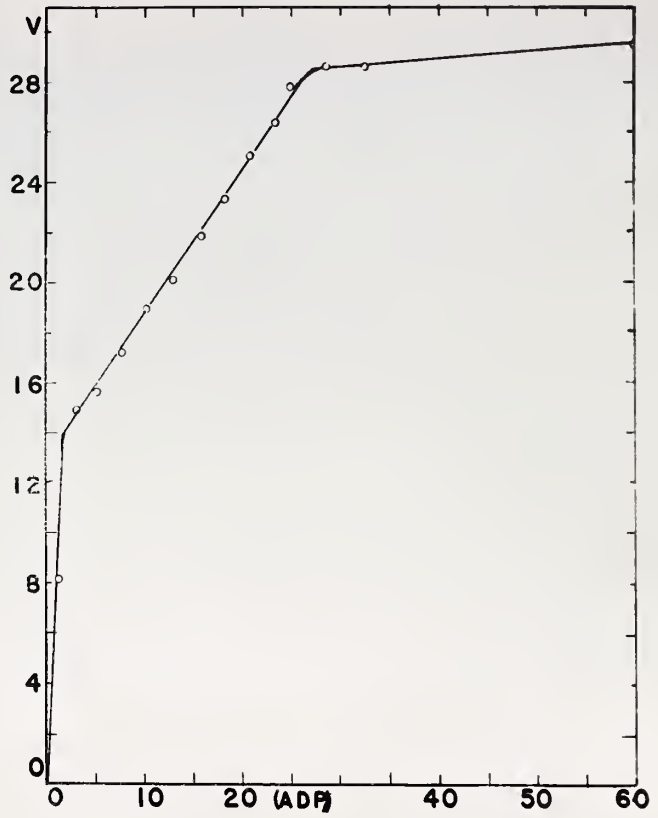


Fig. 3. Effect of ADP Concentration on PPFase Activity.

(e) Effect of DPNH Concentration

TABLE 10
Effect of DPNH Concentration on the Initial Velocity of the Coupled PPFase-LDH System

DPNH Conc. (final) M	Activity (units)
0.00005	20.8
0.00010	20.8
0.00020	21.0

(f) Optimum pH

TABLE 11
The Effect of pH on PPFase Activity

pH (corrected)	Activity (units)
6.0	16.7
6.79	23.8
6.99	27.0
7.32	28.6
7.58	31.0
7.62	30.4
8.01	24.4

The optimum pH is 7.5.

Changing the pH from 7.55 to 6.58 and 7.85 reduced the estimated LDH activity 18% and 6.7% respectively.

(vii) Effect of Preincubation on Na^+ and Ca^{++} Inhibition

TABLE 12
Effect of Incubation on Extent of Na^+ and Ca^{++} Inhibition

Preincubation Time* (min.)	Inhibiting Ion	Conc. of Inhib. Ion	% Inhibition
0	Na^+	0.1	13.6
10	"	"	16.5
20	"	"	21.1
30	"	"	21.0
0	Ca^{++}	0.005	63.6
15	"	"	64.0
20	"	"	65.0
30	"	"	65.0

*Preincubation time refers to time during which the enzyme and inhibitor were incubated in the presence of the activating ions and substrate, prior to both the addition of buffer and DPNH, and the customary 6 minute incubation period. Preincubating the enzyme and inhibiting ion in the absence of substrate and activating ions gave no significant difference in the degree of inhibition.

The 20-minute incubation period gave reproducible results. A series of 4 experiments on PPFase inhibition by 0.001 M Ca^{++} gave values of 34.4, 33.0, 33.0 and 34.6% inhibition. A similar series for inhibition by 0.1 M Na^+ gave values of 30, 31.5, 31 and 27.8% inhibition. There were occasions, however, where the instability of the multiplier photometer did have an adverse effect on the reproducibility. The extent of inhibition is independent of enzyme concentration.

(viii) Effect of ATP*, AMP**, and $HPO_4^=$

TABLE 13
Effect of ATP on PPFase Activity

Experiment No.	ADP Conc. (M)	ATP Conc. (M)	Activity
I	0.0005	--	29.4
	"	0.0013	32.2
	"	0.0005	30.1
	--	0.0013	7.4
II	0.0032	--	21.2
	"	0.0048	21.7

Using either Mg^{++} or Co^{++} it was possible to obtain activity in the absence of ADP, but with ATP present.

Different enzyme concentrations have been used in experiments I and II.

TABLE 14
Effect of AMP on PPFase Activity

Experiment No.	ADP Conc. (M)	AMP Conc.	Activity
I	0.00005	--	21.2
	--	0.0016	0
	0.00005	0.0003	24.4
	"	0.0008	27.8
	"	0.0016	30.3
	"	0.0032	33.3
II	0.0010	--	15.1
	"	0.0016	20.0
	"	0.0032	21.7
III	0.0030	--	23.9
	"	0.0016	26.4
	0.0036	--	24.4

Different enzyme concentrations have been used in experiments I, II and III.

*ATP = adenosine triphosphate
**AMP = " monophosphate (adenylic acid)

TABLE 15
Effect of K_2HPO_4 on PPFase Activity

ADP Conc. (M)	$HPO_4^=$ Conc. (M)	Activity (units)
0.0005	--	23.8
"	0.0004	33.0
"	0.0016	35.7
"	0.0029	34.5
"	0.0042	34.5
"	0.0084	35.6
"	0.0017	34.4

7. Determination of Relative Velocities

As noted previously the range of velocities that can be measured by the optical assay method is somewhat limited. In many of the activation studies it was necessary to hold one of the components (I) of the assay mixture at suboptimum concentration while another component (II) was varied from suboptimum to optimum concentration. The concentration of (I) was then increased towards its optimum value and (II) was again varied over a range of concentrations. As would be suspected it was necessary to change the enzyme concentration every time the increase or decrease in the concentration of (I) resulted in velocities falling outside of the range desired for measurement.

It is possible to run separate experiments using optimum concentrations of all components and so obtain the velocity under optimum conditions of each enzyme concentration used at each suboptimum concentration of (I). Using these maximum velocities it is then possible to recalculate all results obtained at suboptimum concentrations of (I) on the basis of equivalent enzyme concentration. However, it is usually the case that when (I) is at its lowest concentration, the enzyme concentration used must be so high that it cannot be measured accurately under optimum conditions by the optical assay method.

The following data indicate that relative velocities obtained using a single enzyme concentration, and by varying the concentration of (I) to each of the several optimum and suboptimum concentrations used while all other components are held at optimum concentrations, are equivalent to the method of using equivalent enzyme concentrations previously described. The method using equivalent enzyme concentrations is theoretically more sound and has been used wherever possible.

PEPA may be regarded as component (I)in the following table.

TABLE 16
Relative Velocities for Suboptimum PEPA Concentrations
Using a Single or a Series of Enzyme Concentrations

PEPA Conc.* (final) M	A	B	C	% Error
0.00020	18.1	13.2	12.6	4.6
Optimum	38.5	28.5	28.5	
0.00065	27.7	26.6	25.6	3.8
Optimum	29.4	28.5	28.5	

*All other components of the assay were at optimum concentrations. The optimum PEPA concentration is regarded as being 0.00078 M.

Key to Columns A, B and C:

A = velocities obtained at two different enzyme concentrations using both the suboptimum PEPA concentrations being considered and the optimum concentration, the latter giving the maximum velocity at that enzyme concentration.

C = the velocities obtained at these suboptimum and optimum concentrations of PEPA using a single enzyme concentration.

B = velocities obtained in column A recalculated on the basis of equivalent enzyme concentration, using as maximum velocity under optimum conditions, that obtained under similar conditions in column "C", for comparative purposes only.

For inhibition experiments the % inhibition, which is independent of enzyme concentration, within reasonable limits at least, was first determined, using any enzyme concentration which gave measurable velocities for both control and assay containing the inhibitor. The velocities under normal conditions (i.e. in the absence of the inhibitor) were determined using one of the previously described methods, the velocities in the presence of the various inhibitor concentrations being then calculated from the % inhibition.

8. Discussion

The spectrophotometric assay is theoretically more sound than the colorimetric type assay of enzyme activity. It is possible to use the

initial part of the activity curve where the observed rate is a characteristic property of the enzyme. It allows correct measurement of initial velocities with low PEPA and ADP concentrations and relatively high PPFase activity.

It is indeed fortunate, considering the nature of the kinetic studies carried out, that hemoglobin does not bind sodium and potassium ions, at least at pH 7.4. This is in agreement with results of extensive studies made by Carr (76).

Dialysis appears to be an effective method for removing endogenous substrates and metal ions. It has been reported (51) that the addition of 2% albumin to the hemolysate from human erythrocytes was necessary to prevent inactivation of PPFase on dialysis whereas this was an unnecessary prerequisite when rabbit muscle was used as the source of the enzyme. Results obtained using hemolysate from rabbit erythrocytes as a source of PPFase seem to indicate that the addition of albumin prior to dialysis is unnecessary in this case. The apparent anomoly is explicable on the basis of species differences in the two instances.

The addition of excess LDH produced increases in velocity which were considered to be insignificant, especially since they are small and are present in all measurements. No commercial preparations of LDH are yet available which are free of PPFase activity. That LDH is present in excess is also evidenced by the fact that changing the DPNH concentration had no measurable effect on the initial velocity (Table 10). The initial velocities are also proportional to the PPFase concentration.

The optimum concentrations of the activating ions (Tables 6 and 7) compare favorably with those obtained by the colorimetric method (85), the

optimum Mg^{++} concentration being 0.003 M lower and the optimum K^{+} concentration being 0.025 M higher using the colorimetric method. The Mg^{++} requirement showed a wide optimum, and the optimal concentration was selected at its upper limit.

The optimum PEPA concentration (Table 8a) was 0.00078 M as compared to 0.003 M for the colorimetric method. Increasing the concentration to 0.0012 M at K^{+} concentrations of 0.015 M, 0.075 M, and 0.20 M increased the velocity 2.5%, 0% and 2.8% respectively. The K_m for PEPA has been estimated to be 0.0004 M as compared to 0.000086 M reported previously (78a). However, the value of the Michaelis constant depends upon the concentration of ADP. The concentration of PEPA required to saturate PPFase apparently increases with pH.

Between 0.00023 M and approximately 0.0027 M ADP the maximum velocity is proportional to the ADP concentration (Table 9 cf. Fig. 3). Alberty (87) has summarized a variety of mechanisms from the literature found useful for interpreting the mechanisms of coenzyme action. Without considering these in detail it may be stated that the mechanism of ADP interaction in the PPFase system has one of two of the following possibilities:

(a) Mechanism involving two binary complexes

$$E + A \rightleftharpoons EA$$

$$EA + B \rightleftharpoons EC + D$$

$$EC \rightleftharpoons E + C$$

(b) Mechanism involving a single ternary complex

$$E + A \rightleftharpoons EA$$

$$EA + B \rightleftharpoons EAB \rightleftharpoons EC + D$$

$$EC \rightleftharpoons E + C$$

or EAB may dissociate directly:

$$EAB \rightleftharpoons E + C + D$$

The two mechanisms may be distinghished only by transient state studies (dissociation constants have been omitted from the equations for convenience).

As noted by Alberty (88) the Michaelis constant for A determined by using high concentrations of B, will depend upon the rate of dissociation of the EC complex and not upon the rate of dissociation of A from EA.

The optimum pH was estimated to be 7.5 (Table 11) as compared to previously reported values of 7.0 (85) using as source of the enzyme human erythrocytes, and 7.5 (84a), the source of the enzyme not being reported. Unless stated to the contrary all studies in this thesis have been carried out at pH 7.5.

The effect of addition of ATP upon PPFase activity (Table 12) is no doubt due to the active ATPase present in the hemolysate (73). Addition of low concentrations of ATP (Experiment II, table 13) showed little effect upon PPFase activity, so that its effect under normal circumstances is considered to be negligible.

It has been reported that PPFase is capable of transferring "high energy phosphate" from PEPA to guanosine and cytidine diphosphate (89) and also to deoxy ADP (90). There has also been much reference in the literature (91, 92, 93, 94) to the ability of PPFase to transfer phosphate from PEPA to AMP. Bücher and Pfleiderer (78a) attribute such properties to be due to contamination by myokinase. The results obtained by addition of AMP to assays, with and without ADP (Table 14) may be accounted for if it is assumed the hemolysate from rabbit erythrocytes contains a high myokinase activity. No reaction occurs in the absence of ADP, when AMP is present.

No explanation can be offered at present for the activating effect of $HPO_4^=$ (Table 15). The results may have important implications if data using phosphate and other types of buffers are to be compared.

B. A KINETIC ANALYSIS OF CATION INTERACTIONS

1. Introduction

Enzymes have been defined as organic catalysts elaborated by living matter. The term "catalyst" was first proposed by Berzelius in 1836, with reference to reactions such as the thermal decomposition of peroxide by metals and the conversion of starch to sugars. It is recognized today that few enzymatic reactions can be duplicated in the laboratory under conditions approaching those which can be considered as physiological. Although many enzyme reactions have been investigated with the object of elucidating the mechanisms underlying their action, those proposed have been of a speculative and tentative nature, very few definite conclusions having been reached.

The endeavor to elucidate the nature of enzyme catalysis has drawn heavily upon the field of reaction kinetics. Quantitative studies of the kinetics of enzyme action have either centered on studies of the overall reaction (steady-state kinetics), or demonstration of the formation and breakdown of enzyme-substrate complexes directly by means of special techniques (transient state kinetics).

One of the major contributions of kinetic studies to our knowledge of enzymatic action is considered to be the concept of the enzyme-substrate complex. Henri (96) and Brown (97) first proposed that the diphasic nature of the dependence of sucrase activity upon sucrose concentration could be accounted for by the formation of an intermediate complex between the enzyme (E) and substrate (S) which requires a finite length of time to break down

into products. Later Michaelis and Menten (98), using several basic assumptions, formulated the concept of the E-S complex mathematically. They assumed:

(i) the total concentration (E) of potentially active loci is equal to the sum of those coupled with substrate (ES) and those not so coupled (E).

$$(E_t) = (E) + (ES) \qquad (1)$$

(ii) the velocity of reaction is proportional to (ES),

$$\frac{V}{V_m} = \frac{ES}{E_t} \qquad (2)$$

where V_m = maximum velocity; i.e. all loci are saturated with substrate.

(iii) as a reversible reaction

$$E + S \rightleftharpoons ES \qquad (3)$$

the enzyme substrate coupling is subject to the mass law

$$\frac{(E)\ (S)}{(ES)} = K_s \qquad (4)$$

(iv) the thermodynamic activity of the substrate can be represented by its total concentration.

(v) that for the sucrase reaction the concentration of water remained unchanged.

Using these assumptions, they derived the following equation:

$$\frac{V_m}{V} = \frac{E_t}{(ES)} = 1 + \frac{K_s}{(S)} \qquad (5)$$

Lineweaver and Burk (99) have presented a simplified graphical analysis of experimental data according to the Michaelis-Menten theory using the property that the plot of $\frac{1}{V}$ vs. $\frac{1}{(S)}$ is linear, when the theory is applicable.(86).

The rather extensive development of the Michaelis-Menten theory since first proposed has recently been subject to re-examination and elaboration (100, 101, 87). The steady-state treatment which has been further developed and applied in a general manner by Freidenwald and Maengwyn-Davies (100) will be employed to a considerable extent in the study of cation interactions in the PPFase system. The steady-state treatment is applicable when the rate of association and dissociation of the E-S complex is very fast, as compared to its rate of decomposition into enzyme and products. Also, by holding certain concentration variables constant, it is possible to apply rate equations derived for very simple mechanisms to much more complicated enzyme systems, the simple equations being special cases of much more complicated mechanisms.

Freidenwald and Maengwyn-Davies have developed a generalized Michaelis equation for cases in which one molecule of each of substrate (S), activator (A), or inhibitor (I), combines with each active locus of the enzyme; i.e. a first-order theory. They include in equation (1) above, the additional molecular species (EI), (EA), (EAI), (EIS), (EAS) and (EAIS). Applying first the mass law, then the other concepts of the Michaelis and Menten theory, they obtained the following equation:

$$\frac{V_m}{V} = 1 + \frac{xK_sK_A}{(A)(S)} + \frac{xK_A}{(A)} + \frac{xK_S}{(S)} + \frac{xK_SK_A(I)}{K_I(A).(S)} + \frac{xK_S(I)}{K_I(S)} + \frac{xK_A(I)}{\alpha K_I(A)} + \frac{(I)}{\alpha K_I} \qquad (6)$$

K_s = Michaelis constant; K_I and K_A are dissociation constants of the I-E and A-E complexes.

Two constants, both of little practical use, have been omitted. Rearranged forms of this equation, easily amenable to graphical analysis, are presented by the authors.

α and x are dissociation factors.

$$\frac{(EI)(S)}{(EIS)} = \alpha K_S \quad \text{and} \quad \frac{(ES)(I)}{(EIS)} = \alpha K_S$$

The inhibition is non-competitive, competitive or anti-competitive (or coupling) when α has the values 1, ∞, and 0 respectively. Similarly:

$$\frac{(EA)(S)}{(EAS)} = x\ K_S \text{ and } \frac{(ES)(A)}{(EAS)} = x\ K_A\ .$$

According to the values of x "non-coupling" and "coupling" (two types) activation are distinguishable.

In the special case where the inhibitor complexes with the substrate, the "free" (S) not "total" (S_t), substrate concentration must be used and may be calculated by use of the following equation:

$$S = (S_t) - \frac{x}{2} \frac{1}{2} (x^2 - 4(S_t).\ I_t)^{1/2} \qquad (7)$$

where $x = (S_t) + (I_t) + K_{SI}$

It is important that the dissociation coefficient of the S-I complex be known from non-enzymologic measurements since there is danger in interpreting such inhibition as competitive inhibition.

Also in the special case where the activator couples with the substrate, it is desirable to know the dissociation constant of the S-A complex from non-enzymologic measurements.

Linear plots of $\frac{1}{V}$ vs. $\frac{1}{(S)}$ are to be expected in the presence of both A and I if only the substrate concentration is varied. Similarly if only (A) or (I) is varied, linear plots are to be expected for $\frac{1}{V}$ vs. $\frac{1}{(A)}$ and $\frac{1}{V}$ vs. (I).

The second-order theory, where two molecules of the same substance may be coupled per active locus of the enzyme, is developed. The second-order theory is used to describe inhibition by excess substrate and activator. Either (S) or (A) is substituted for (I) in equation (6); (A) in this case

is usually replaced by the non-comittal symbol (M). V vs. log (M) or log (S) should yield a symmetrical curve, and $\frac{1}{V}$ vs. $\frac{(Mo)}{(M)} + \frac{(M)}{(Mo)}$ should give a straight line if the theory is applicable. (Mo) = concentration of the activator which yields maximum activity.

The effect of pH on enzymatic activity has usually been subject to special treatment (87, 100, 102, 103). The investigation of the effect of pH is based on the view that pH dependence is due primarily to the ionization of groups in the enzymatic site. Since proteins are polybasic acids, the effect of pH on kinetics could become very complicated, but there is reason to believe some idea may be gained of the ionizable groups which are closely connected with enzyme activity.

In order to account for a pH optimum in the maximum initial velocity it is necessary to postulate at least two ionizable groups. Alberty (103) has inferred from the kinetic data for fumarase action that the catalytically active form of the enzyme is that in which one essential group has a proton on it, and the other does not. It is also interesting to note that the type of buffer used may be of importance since phosphate buffer showed an activating effect on fumarase.

Dixon (102) has calculated the various ionization constants from plots of - log K_S and - log K_I vs. pH. Friedenwald and Maengwyn-Davies (100) evaluate the constants from a plot of $\frac{1}{V}$ vs. $\frac{(H_o^+)}{(H^+)} + \frac{(H^+)}{(H_o^+)}$ where (H_o^+) = hydrogen ion concentration at optimum pH. This applies only to the second order theory (not higher orders).

Experimental facts seem to indicate (100) that the pH optimum curves, in cases where one species of substrate ion is present, are nearly symmetrical. This is true in most cases where the substrate is a strong acid or base or

is unionized. Friedenwald and Maengwyn-Davies (100) also point out that since there are many enzyme systems for which it has been shown that the pH optimum and shape of the pH vs. activity curve does not change markedly with substrate concentration, then K_{si} must remain more or less constant:

$$K_{si} = \frac{(E_i)(S)}{(\overline{E_i})(S)} \quad \text{where } E_i = \text{an ionic species .}$$

They postulate enzyme catalysis therefore to be, in the main, an all or none affair.

Their analysis further indicates that for non-coupling, non-competitive hydrogen ion effects the activity vs. pH curve should merely change in height with a change in (S), the K_{si} being independent of pH. This indicates that an ionic groups essential for coupling are sufficiently strong acids as to not have their dissociation significantly suppressed. Competitive inhibition will result when affixing a hydrogen ion at an active locus will interfere with binding of the substrate. In this case the K_{si} will change and the V vs. pH plot will be assymetrical, steeper on the low pH side. Further a plot of K_{si} vs. (H^+) should give a straight line.

The situation becomes more complicated if there are several ionic species of substrate present in the pH range investigated and the variation of the apparent Michaelis constant with pH will depend upon the number of ionic species present. Much more complicated forms of the Michaelis-Menten equation are obtained when the rejected ionic species act as competitive or non-competitive inhibitors.

Alberty (103) recognizes that the interpretation of pH data is subject to many pitfalls. Although ionizable groups at the active locus will exert the preponderant effect on catalysis through ionization, groups

in the immediate vicinity of the active locus and possibly all the rest of the groups of the molecule will exert at least electrostatic effects on other groups in the catalytic site. Corrections should be made for any denaturation, ionization of substrate, or buffer effects which occur with changes in pH.

The pH dependence of metal ion-binding to proteins (105) and the effect of pH on the interaction of enolase with activating metal ions has been studied (106) in order to identify the group involved in binding the metal to the protein or enzyme. In the latter case the changes in dissociation constants of the metal-enzyme complex with changes in pH are helpful in characterization of the active site of the enzyme.

There have been a number of general mechanisms of enzyme action proposed. They may be summarized briefly as: (a) strained molecule theory of Quastel (108a), later modified by Pauling (108b) in which he suggested that the active site was a perfect fit for the strained molecule; (b) Haldane's theory of hydrolytic enzymes as acid base catalysts (108c) which function by increasing the normal sensitivity of the substrate to hydrogen or hydroxyl ions; (c) theory of polyfunctional catalysts proposed by Swain and Brown (108d) whereby an acid and basic group at the active site serve to bind and act upon the substrate; (d) the transfer of protons or electrons to or from the substrate as a result of tautomeric changes in the protein (enzyme) molecule. Such theories can only serve as models with which to interpret results of kinetic studies.

Lehninger (48) suggests that metallo-enzymes may offer the best approach to a solution of the problems underlying enzyme catalysis, since quite often catalytic activity is to be found in a primitive form in the

simple inorganic ions themselves. Studies of heme enzymes seem to indicate that the function of the protein moiety may be to enhance the properties of the metal.

In order to explain metal ion specificities and antagonisms it is suggested that structural requirements of the ions should be studied. Parameters of ion structure and properties such as mass, ionic charge, and ionic radius, configuration and stability of hydrates of metallic ions in solution, and configuration and stability of coordination complexes with substrates and enzymes are of considerable importance. With respect to factors influencing the stability of chelates or complexes between metal ions and chelating or complexing agents, Calvin (105b) has sought to divide them into two groups. Those factors which are related to the stability of complex compounds in general, not limited to the stability of chelate compounds, are first the charge, radius, and available orbitals of the metal, and secondly the size (steric repulsion), polarizability, and basicity of the complexing group. Those factors which are related to the stability of chelate compounds only, include steric effects, entropy effects, and resonance effects. It has proven impossible to explain ion specificity on the basis of crystal radii, and the factors of hydration and complex formation are areas which have not been adequately explored. Data regarding the stability of physiologically important ions with enzymes and substrates are particularly limited. At present the approach has been to study each enzyme individually with respect to its metal requirements, and to avoid generalizations.

As pointed out by Lardy (109) there is apparently an absolute requirement for inorganic ions in every enzymatically catalyzed transphosphorylation reaction involving the adenylic system; however the only generalization that

can be made as yet is that specific divalent cation requirements are met by relatively low concentrations while univalent cation requirements (if required at all) are met by relatively high concentrations.

The divalent ions, e.g. Mg^{++} or Mn^{++}, are often thought of as binding the substrate or coenzyme to the enzyme, thus serving as a bridge between anionic groups. Martell and Calvin (110) believe that these two ions are bound to the enzyme through chelate rings. They base their belief on the fact that the use of deactivators has shown Mg^{++} to have two free valencies when bound to carboxypeptidase. This indicates that the two remaining covalencies of normally tetra covalent Mg^{++} must be bound to the protein, and therefore are probably involved in chelation. While in general five-membered chelate rings are favored, four- and six-membered rings are also possible; any of these are very unlikely. In addition, metals such as cobalt preferentially bond to nitrogen donors while ions of more basic metals such as magnesium are more greatly attracted to oxygen. Manganese, intermediate between these extremes, will bond to both.

Recent physico-chemical measurements on the stability of ionic complexes of various adenosine phosphate compounds with divalent and univalent cations (111a, 111b, 112) will no doubt contribute to the understanding of the action of metal ions in enzyme systems where such phosphate compounds are utilized as coenzymes. Van Wazer et al. (113) have found the complex-forming capacity of a polyphosphate to be approximately proportional to the number of phosphorus atoms present in the chain. Melchior (112) believes that it is through complex formation with ATP that Na^{+} and K^{+} may exert their effect on enzymatic reactions.

The technique of nuclear magnetic resonance recently applied to

chemical problems may prove to be a useful tool in studying the binding of ions to substances important in biological systems. It has been used by Jardezky and Wertz (114) to demonstrate that sodium forms specific complexes with lactate, pyruvate, and citrate even in relatively dilute solutions (0.1 M).

A new approach to the study of the action of metal ions in enzyme systems has been the use of metal buffers. Using the pCa^{++} activity curve of phosphatase from potatoes and known dissociation constants of the Ca^{++}-ADP complex, Raaflaub (115) concluded the studied reaction had a Ca-protein complex as intermediate.

In view of the nature of the present kinetic study the results of recent investigations on two metal-requiring enzymes is of practical interest. Malmström (116) studied the metal ion specificity in the activation of enolase, the enzyme which forms PEPA from 2-phosphoglyceric acid in the glycolytic mechanism. The conclusion is drawn that to activate enolase an ion must be able to combine with the enzyme and substrates and must have a certain relation between charge, size and structure of its complex with substrates to allow formation of an ES complex.

Morrison et al. (117) have made a study of the kinetics of the reactions catylyzed by arginine phosphokinase, a phosphate transferring enzyme. The conclusion was reached that Mg^{++} ions react with arginine phosphokinase to form an active Mg-arginine phosphokinase complex which in turn reacts with the free forms of ATP and ADP. They also concluded that ATP and ADP were bound to the enzyme at the same site.

Caution is required in proposing any reaction mechanism for, as Segal et al. (101) point out, if results of an experiment on an enzyme fit a postulated mechanism, it can only be concluded that such a mechanism is one possibility, and as much collateral evidence as is possible to attain should be used in support of it. Facts from experiments, according to

Alberty (87), serve to describe further details of the mechanism, but like absolute zero the complete mechanism may be approached but never reached, at least until the chemical nature of the catalysts is known.

The results of the kinetic analysis are reported and interpreted graphically. In all cases only two variables in the system were varied at a time; i.e. the concentration of an activator and substrate, an inhibitor and substrate, or of an inhibitor and an activator*. Since Lineweaver-Burk (L-B) plots give too much weight to the lower concentrations (118) and since binding by components of the reaction mixture can have significant effects on velocities in the lower concentration ranges of one of the components so affected, much less weight has been placed on values in this region of the plot.

Preliminary studies on PPFase with the colorimetric method of measuring activity include K^+ activation (47, 49), inhibition by excess activators (49), Ca^{++}-K^+ antagonism (47) and Na^+-K^+ antagonism (49). These studies have been reinvestigated and extended using the spectrophotometric method.

2. The Kinetics of Activation of PPFase

Kachmar and Boyer (47) from a kinetic consideration of a number of possible mechanisms for K^+ activation of PPFase concluded that there is an independent combination of K and PEPA with the enzyme under equilibrium conditions to form an active ternary complex.

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*Except for the variables, the concentration of all other components in the reaction mixture were held at optimum concentrations, except for ADP where a concentration of 0.0005 M was used.

Results of K^+ activation studies with varying concentrations of the substrate (PEPA) are in accord with those previously reported. The plot of 1/V vs. $1/(K^+)$ for varying PEPA concentrations* (Fig. 4, Table A-1) shows linearity in the higher concentration ranges and has a common intercept. This indicates that the Michaelis constant for K^+ i.e. K_a is constant, therefore independent of (PEPA). The coordinates of the intercept on the abscissa are -1/Ka and $1/V_m^{(1-x)}$; thus x is equal to one. Freidenwald and Maengwyn-Davies (100) speak of the situation where x = 1 as "non coupling activation." Such an activator when attached to the enzyme has no effect on the affinity of the enzyme for the substrate, and conversely the attachment of the substrate to the enzyme has no effect on enzyme-activator association. It may be further postulated that since activation by K^+ follows the 'first order theory' then only one potassium ion need be bound per active enzyme locus (i.e. per PEPA molecule bound) for activation of PPFase.

The reduction in velocity as the (K^+) is increased to concentration values in excess of the optimum, indicates inhibition by excess activator, and an analysis of the nature of such inhibition will be attempted later by means of the 'second order theory' according to Freidenwald and Maengwyn-Davies.

Kachmar and Boyer (48) compared the relative activating capacities of K^+, Rb^+ and NH_4^+ and found them to be in the ratio 1.00 : 0.84 : 0.72. They also reported that, in the absence of K^+, Na^+ had a real but weak activating capacity, but Li had no such property. In the presence of very high hemolysate concentrations it has been possible to run a V vs. Na^+ activation plot (Fig. 5, Table A-2). There is a rather sharp optimum around

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*V = units of PPFase activity

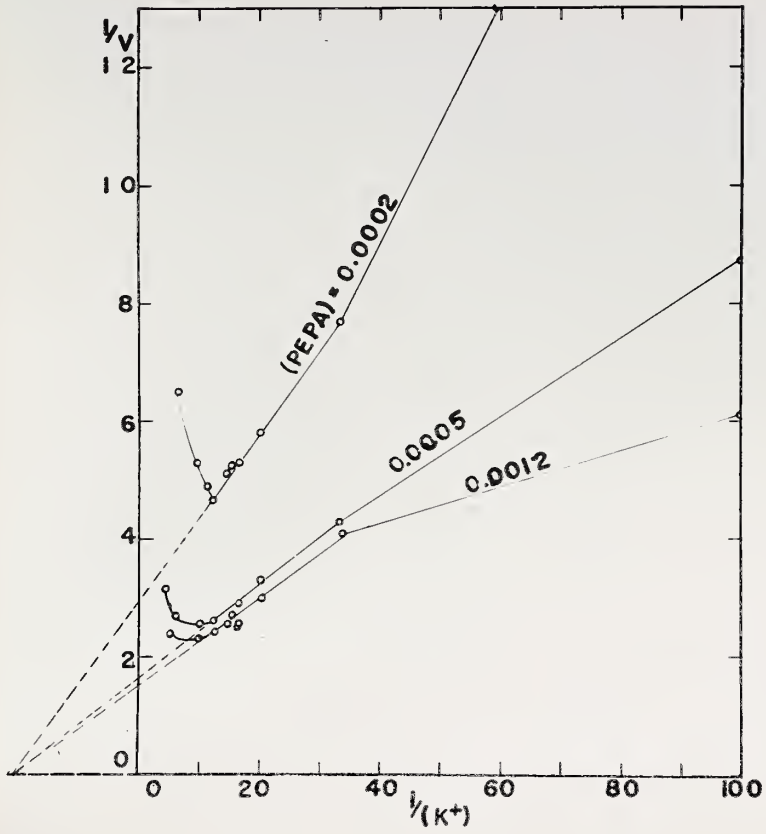

Fig. 4. K^+ Activation of PPFase with varying (PEPA).
LB plot of 1/V vs. $1/(K^+)$

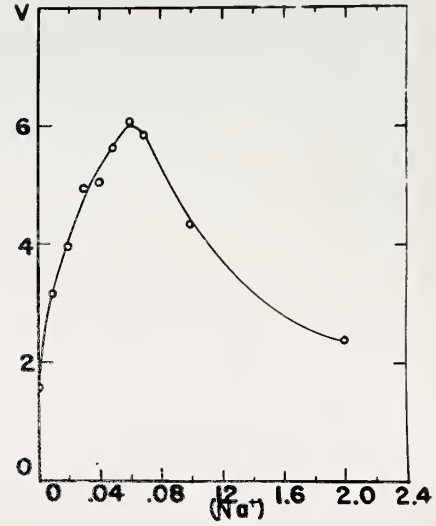

Fig. 5. Na^+ Activation of PPFase using high enzyme concentrations.
Plot of V vs. (Na^+)

0.06 M which is of the same order as that of K^+. The apparent initial velocity in the absence of any added Na^+ can be attributed to a small amount of K^+ present in the Na^+, K^+, salt of ADP.

The adenosine phosphates are exceptions to the rule that a coenzyme acts catalytically and are now often treated as substrates in the literature. It is of advantage to treat ADP as a "second substrate" in the present analysis. A kinetic analysis of activation of PPFase by univalent cations has also been carried out with the (ADP) as variable, while the concentration of the "first substrate" (PEPA) was held constant at its optimal value. The L-B plots for 1/V vs. $1/(NH_4^+)$ with varying ADP concentrations (Fig. 6, Table A-3) and for 1/V vs. $1/(K^+)$ with varying ADP concentrations (Fig. 7, Table A-4) indicate that K^+ and ADP also combine with the enzyme independently of one another, i.e. non coupling activation. Excess NH_4^+ also causes inhibition. Ka values for K^+ and NH_4^+ are estimated to be 0.050 M and 0.036 M respectively.

The order of relative activation by K^+, NH_4^+ , Rb^+ and to some extent Na^+, and inhibition by Na^+ and Li^+ led Kachmar and Royer (47) to a comparison of their physical properties. They concluded, after examining data available in the literature, that the estimated radius of hydration, the ionic radius from crystallographic data, and the relative ion mobility, are alike for the three activating ions and distinct from those of Na^+ and Li^+. They postulate the possibility that Na^+ and Li^+ may not be bound as strongly by PPFase as the activators, or that geometric displacement which results from binding of cations by PPFase is different for Na^+ and Li, than for K^+, NH_4^+, or Rb^+.

It may be of some significance that the relative tendencies of cations to be adsorbed by a cation exchange resin can be directly correlated with the magnitude of the charge - radius ratio for the hydrated ions (119).

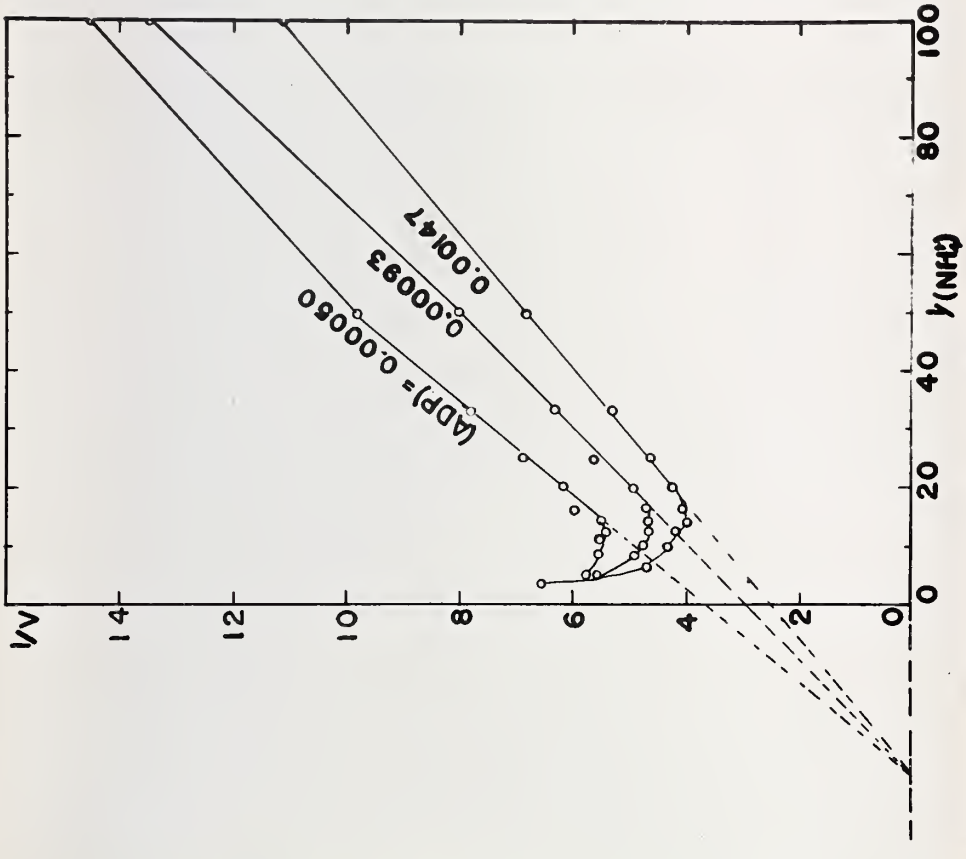

Fig. 6. NH_4^+ Activation of PPFase with Varying (ADP). L-B Plot of 1/V vs. $1/NH_4$.

For the alkali metals the adsorbability decreases in the order Cs^+, Rb^+, K^+, NH_4^+, and Na^+. However this is not the exact order of their relative activities. The activating cations have a lower ionic potential and therefore have less tendency to hydrate or form complexes (48), accounting for the higher charge-radius ratio of the hydrated ions.

The possibility that the activating ions can make more direct contact with the enzyme itself than do the inhibitors, finds some support in the theory of Seifriz (120), in which he interprets the greater toxicity of Rb^+ as compared to Na^+ as being due to its greater activity and its ability to make more direct contact with the protoplasm.

It is evident that some factors must operate in the enzyme molecule which make it possible to distinguish, to a significant extent, univalent ions from divalent ions. It is also evident from a study of univalent ion requirements in the PPFase system that the activation by univalent ions must be due to binding of the activating ion by the enzyme molecule and not a mere influence on the ionic environment which has been suggested as a more or less partial or alternative explanation (121).

Although the univalent cation requirement appears to be a fundamental property of PPFase (60), it is not a general phenomenon in the phosphate-transferring enzymes. However PPFase like most phosphate-transferring enzymes shows a requirement for Mg^{++} (replaceable by Mn^{++} or Co^{++})in the PPFase system. This requirement is obligatory for PPFase and a kinetic analysis has been carried out to determine the nature of its activation.

The L-B plot of 1/V vs. $1/(Mg^{++})$ at four different concentrations of ADP (Fig. 8, Table A-5) suggests that the activation by Mg^{++} with respect to this substrate is of the non-coupling type. Results of a similar plot with Mn^{++} as activating ion (Fig. 9, Table A-6) serve to substantiate the

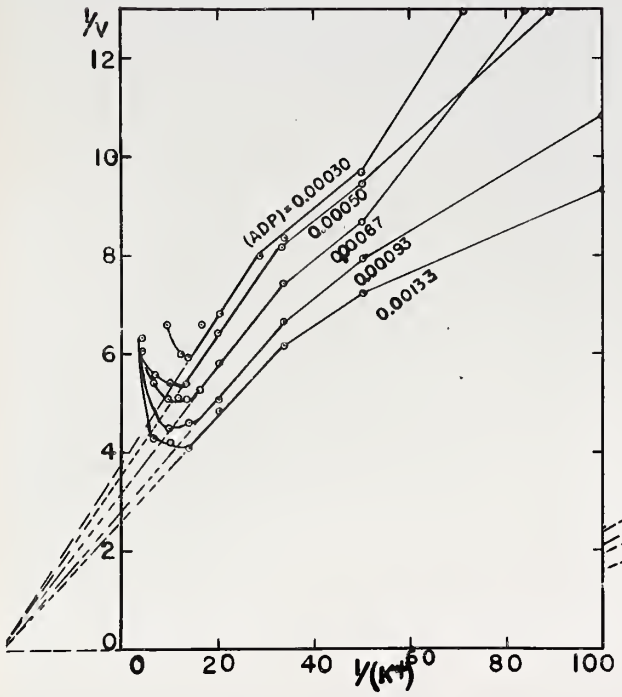

Fig. 7. K^+ Activation of PPFase with varying (ADP).
L-B plot of 1/V vs. $1/(K^+)$

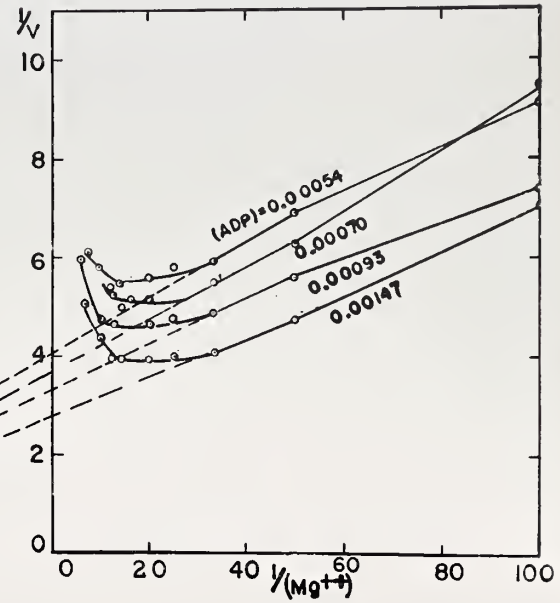

Fig. 8. Mg^{++} Activation of PPFase with varying (ADP).
L-B plot of 1/V vs. $0.1/(Mg^{++})$

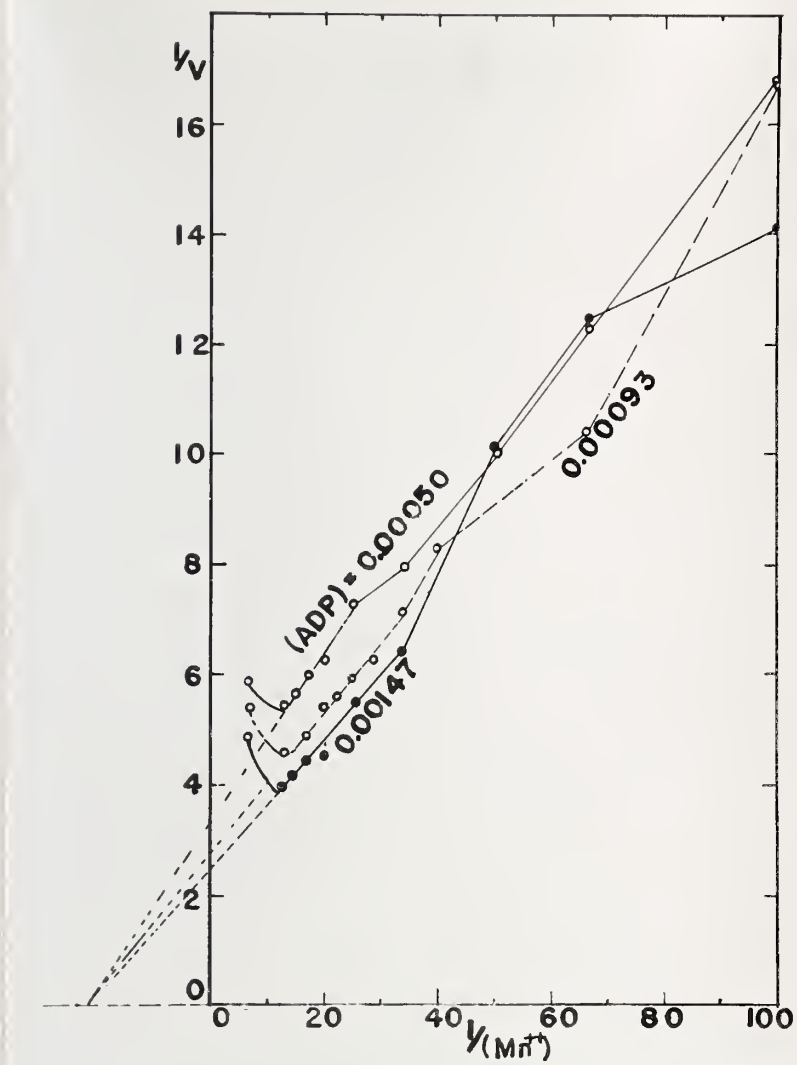

Fig. 9. Mn^{++} Activation of PPFase with Varying (ADP) L-B plot of 1/V vs. $1/Mn^{++}$

fact that the coupling of Mg^{++} or Mn^{++} and ADP with the enzyme is independent; the Michaelis constants remain constant. The nonlinearity of low (Mn^{++}) and high (ADP) may be attributable to binding of Mn^{++} by ADP. Since the stability constants of Mg-ADP complexes are lower than those of Mn-ADP complexes (111b) the binding may not produce such noticeable effects in the former case.

The reduction in velocity which results when the (Mg^{++}) is increased beyond optimal requirements can also be classified as inhibition by excess activator and will be considered later. The Ka for Mg^{++} and Mn^{++} have been estimated to be 0.005 and 0.0046 respectively.

The relation of activation of PPFase by divalent ions to the other substrate, PEPA, has also been studied. The L-B plot for 1/V vs. 1/(Mg^{++}) at varying PEPA concentrations (Fig. 10, Table A-7) proves to be a perfect example of what Friedenwald and Maengwyn-Davies refer to as 'coupling activation of the first type'. Such activation requires that the coupling ion be already attached to the enzyme before the substrate will couple with it. In such a case x approaches zero. Mg^{++} no doubt acts by becoming chelated. The inhibition by excess Mg^{++} is markedly reduced by increasing the (PEPA).

As an alternative to the previous analysis the plot of 1/V vs. 1/(PEPA) with varying (Mg^{++}) is also presented (Fig. 11, Table A-8). Nonlinearity, steeper slope and eventual crossing over of the lines at higher (Mg^{++}) and lower (PEPA) indicate that the velocities in these regions are lower than predicted by the state analysis of Michaelis and Menten. However, at lower (Mg^{++}), i.e. 0.0015 M, the L-B plot approaches linearity. An approximate extrapolation of the lines at high (PEPA) seems to confirm the results of the previous analysis.

Unfortunately no equations to be found in the literature cover the situation where the 1/V vs. 1/(A) plot is linear while the 1/V vs. 1/(S)

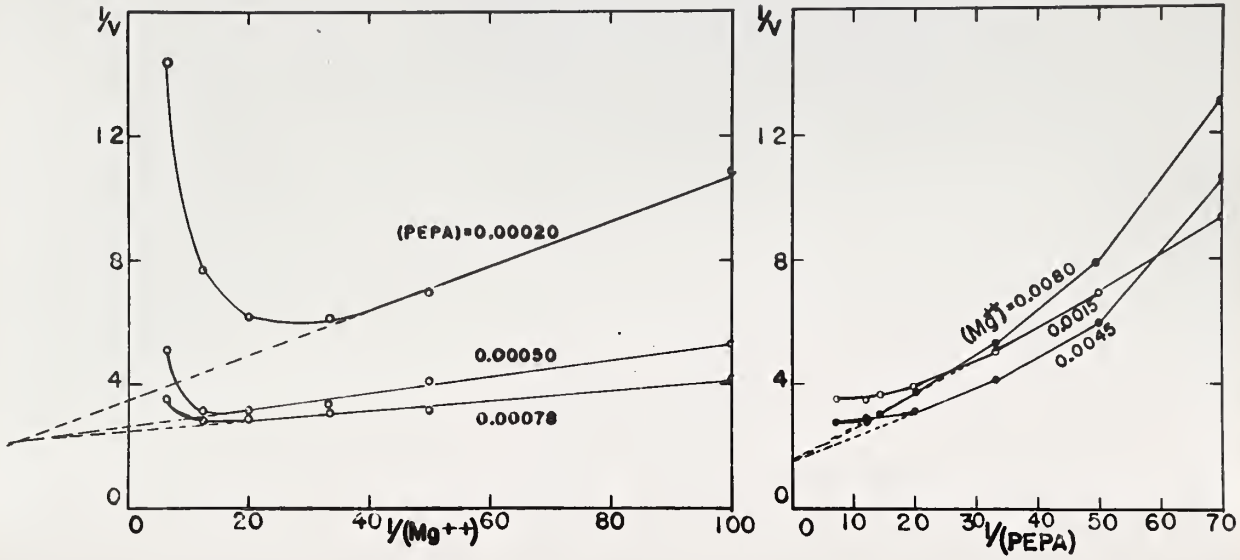

Fig. 10. Mg^{++} Activation of PPFase with varying (PEPA).
L-B plot of 1/V vs. $0.1/(Mg^{++})$

Fig. 11. Mg^{++} Activation of PPFase with varying (Mg^{++}).
L-B plot of 1/V vs. 0.01/(PEPA)

plot is non linear*, and further which will also include the restriction that the activation is coupling activation of the first type. It is apparent that some form of competition must exist between PEPA and a second Mg^{++} ion for the active locus. The very significant increase in velocity obtained by increasing the PEPA concentration (Fig. 10) suggests inhibition by excess Mg^{++} is of the same nature as that which will be tentatively referred to as competitive inhibition by Mg^{++} at suboptimum (PEPA).

The inhibition by excess Mg^{++} and K^{+} has been studied previously by Collier and McRae (49) who used the method of Freidenwald and Maengwyn-Davies as developed within the concept of the 'second order theory.' They found that the V vs. log (Mg^{++}) plots were symmetrical, while the V vs. log (K^{+}) plots were non symmetrical. Similar studies have been carried out with Mn^{++} (Fig. 12) and NH_4^{+} (Fig. 13) at a relatively wide concentration range above and below the optimum**. The V vs. log (Mn^{++}) plot is symmetrical while the V vs. log (NH_4^{+}) plot is asymmetrical.

In the case of inhibition by excess Mn^{++} or Mg^{++} it appears to be a perfect example of an activator forming an activated complex EM, where M represents the divalent metal ion, and which is capable of forming the inactive complex EM_2 at higher concentrations. The symmetrical plot seems to eliminate the possibility that inhibition by excess Mg^{++} is through coupling with the substrate, which is the other possible explanation for the phenomenon encountered.

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* A = activator S = substrate.

** Separate tables have not been presented for Fig. 12 and Fig. 13 since primary data are readily obtained from other tables.

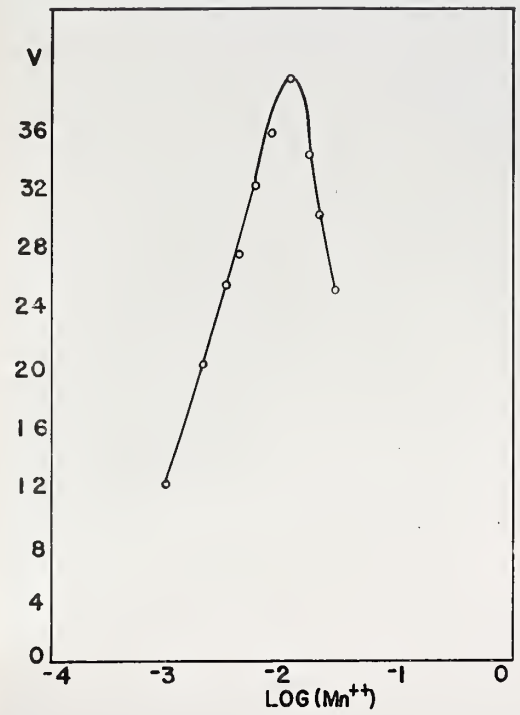


Fig. 12. Analysis of Inhibition by Excess Mn^{++} by the 'Second Order Theory' Plot of V vs. log (Mn^{++})

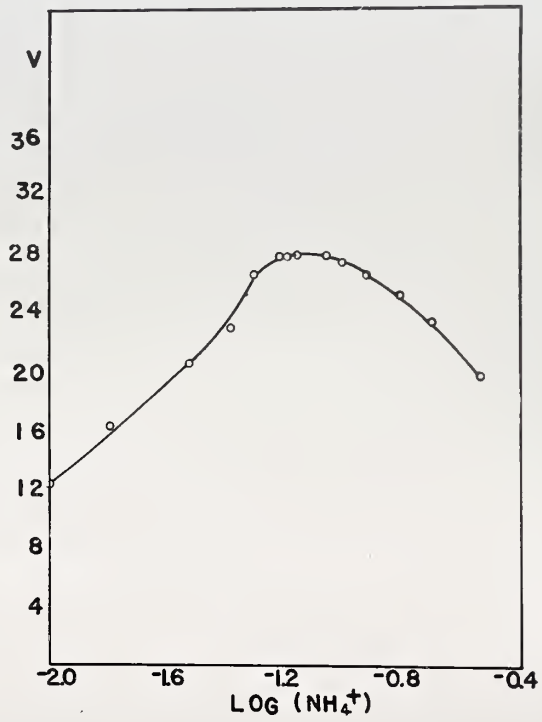


Fig. 13. Analysis of Inhibition by Excess NH_4^+ by the 'Second Order Theory' Plot of V vs. log (NH_4^+)

Although Freidenwald and Maengwyn-Davies present a theory which can be used to determine whether the inhibition is competitive or non-competitive, a linear 1/V vs. 1/(PEPA) plot with varying (Mg^{++}) is required and which is not available in this case. As an alternative, percent inhibition has been determined at concentrations above those considered to be optimum, and concentrations of Mg^{++} in this range have been treated as equivalent concentrations of an inhibiting ion. It is conceivable that once sufficient Mg^{++} is present to activate the enzyme fully, the addition of more activator cannot further activate the enzyme, and therefore it can be treated as a separate species.

The plot of 1/V vs. molar excess Mg^{++} (where molar excess of Mg^{++} represents the amount of Mg^{++} present in excess of the optimal requirement 0.008 M), at varying PEPA concentrations (Fig. 14, Table A-9), resembles competitive inhibition. The deviation of the solid line from the dotted line at 0.00040 M and 0.00078 M can no doubt be explained on the assumption that the percent inhibition is so small that it falls within the experimental error. It is unfortunate that a concentration of 0.0003M PEPA was not used.

The plot of 1/V vs. molar excess Mg^{++} with varying ADP concentrations (Fig. 15, Table A-10) suggests that excess Mg^{++} has very little effect on the participation of ADP in this system. Increasing the (ADP) reduces inhibition by excess Mg^{++} in the lower concentration ranges of the latter by decreasing its thermodynamic activity through complex formation.

It seems quite possible therefore that the second ion of Mg^{++} is competing with PEPA for some point in the active locus of the enzyme.

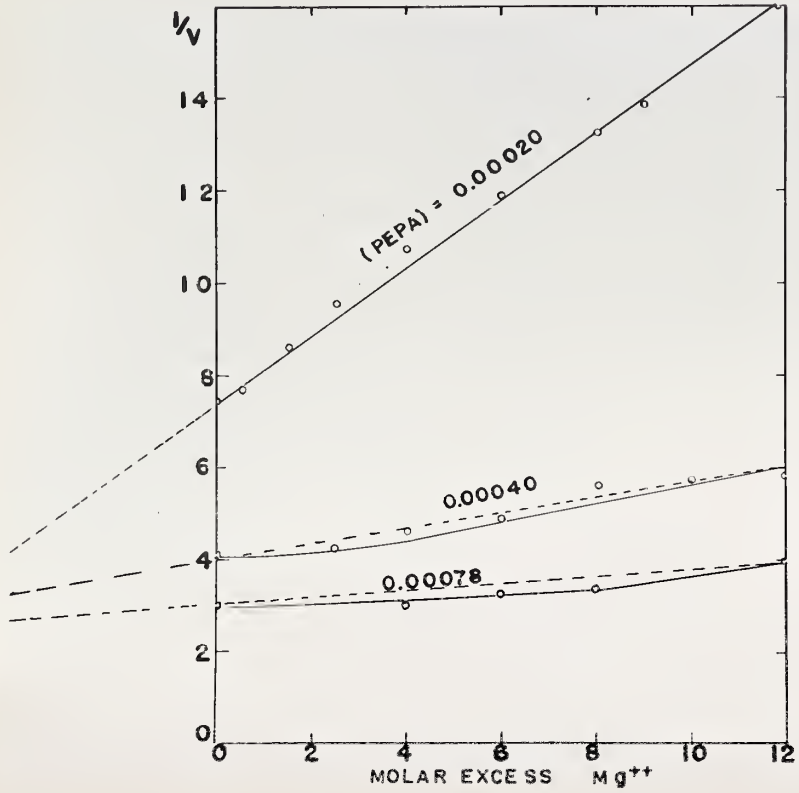
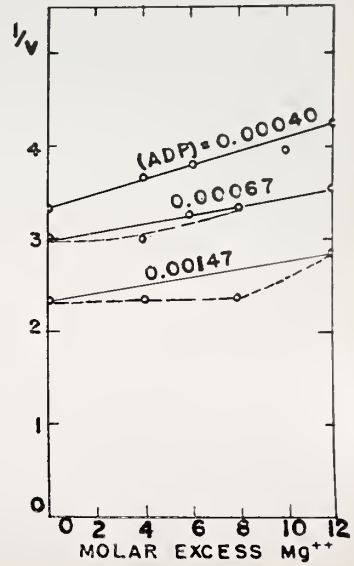


Fig. 14. Inhibition by Mg^{++} in Excess of Optimum with Varying (PEPA). L-B plot of 1/V vs. molar excess Mg^{++}



.. Fig. 15. Inhibition by Mg^{++} in Excess of Optimum with Varying (ADP). L-B plot of 1/V vs. molar excess Mg^{++}

The asymmetry of the V vs. log (NH_4^+) curve suggests that some other mechanism is operative here. The % inhibition at (K^+) in excess of 0.075 M has been determined and plots of 1/V vs. molar excess K^+ with varying PEPA concentrations (Fig. 16, Table A-11) and varying ADP concentrations (Fig. 17, Table A-12) have been made. It is quite evident that at least the major part, and possibly all, inhibition by excess K^+ is due to coupling with the substrate, PEPA. Any coupling with ADP does not appear to produce a significant rate-limiting effect. The nonlinearity of the 1/V vs. molar excess K^+ plots is due to complexing of K^+ with PEPA.

The divalent ions Mn^{++} and Co^{++} are less efficient activators than Mg^{++}. The effect of adding Mn^{++} to reaction mixtures containing optimum concentrations of Mg^{++} has been tested (Fig. 18, Table A-13). The Mn^{++} has an activating effect at lower concentrations of PEPA and an inhibiting effect at higher concentrations. The overall effect is to decrease the Michaelis constant and the maximum velocity. Mn^{++} cannot be treated as an inhibitor with anti-competitive action, as Friedenwald and Maengwyn-Davies point out, an inhibitor which increases the association of the enzyme and substrate should give a plot for 1/V vs. 1/(S) in which the family of lines tend to meet in the lower left hand quadrant.

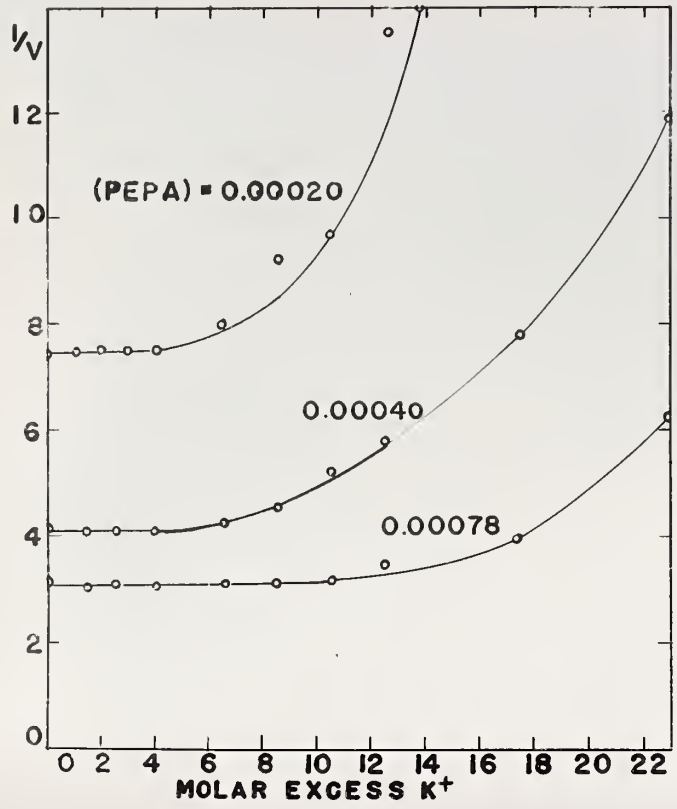


Fig. 16. Inhibition of PPFase by (K^+) in Excess of Optimum* with Varying PEPA.
L-B plot of 1/V vs. Molar Excess K^+

*i.e. @ (K^+) in excess of 0.075 M

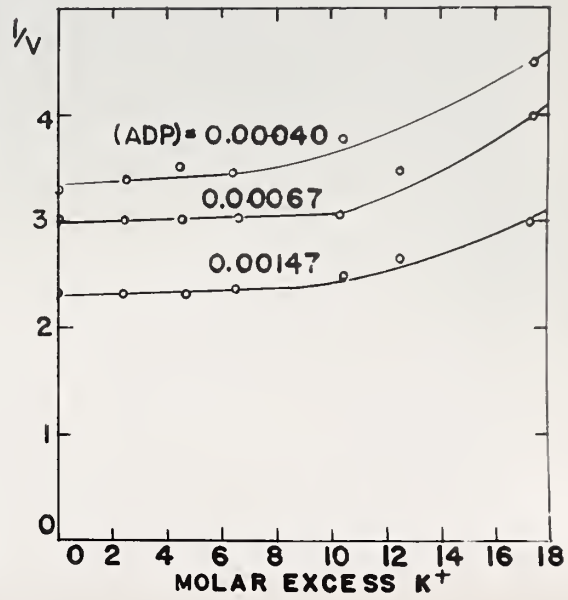


Fig. 17. Inhibition of PPFase by K^+ in Excess of Optimum with Varying ADP.
L-B plot of 1/V vs. Molar Excess K^+

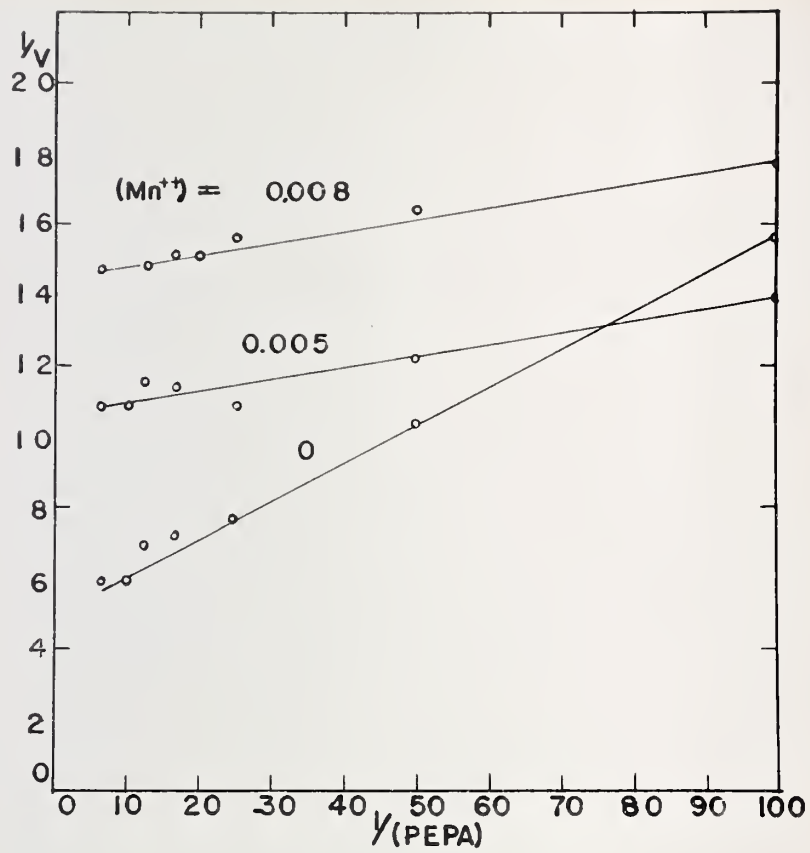


Fig. 18. Effect of Mn^{++} on Mg^{++} Activation of PPFase. Primary and Secondary Data for 1/V vs. 1/(PEPA).

3. The Kinetics of Inhibition of PPFase

The study of the influence of inhibitors on enzyme action is one of the most potent tools available for obtaining information about the active sites of enzymes. It is usual to distinguish two types of inhibition, competitive and noncompetitive. Competitive inhibition is especially useful from the standpoint of learning something of the nature of the enzymatic site.

Noncompetitive inhibition is that type of inhibition in which both the intercept and slope of 1/V vs. 1/(S) plot are increased by the same factor. The effect of a noncompetitive inhibitor is not reduced by increasing the substrate concentration, the maximum initial velocity is lowered, and the Michaelis constant remains constant. Noncompetitive inhibition is usually attributed to combination of the inhibitor at a site neighboring the active site, where it exerts its total inhibitory effect.

Scholefield (123) believes that true competitive inhibition obtains only when the following conditions are fulfilled:

(i) the maximum velocity is unaltered,

(ii) the slope of the 1/V vs. 1/(S) plot is directly proportional to the concentration of the inhibitor.

A competitive inhibitor has been conceived as one which occupies a site in the active locus of the enzyme normally occupied by the substrate. Segal et al. (101) point out that another plausible mechanism can give the same result as competitive inhibition, i.e. apparent competitive inhibition where the inhibitor may only depress the extent of complexing of the substrate with the enzyme, but does not reduce the rate of breakdown of the complex. Other types are found, notably complex formation of the inhibitor

with the substrate, which has already been encountered in dealing with inhibition by excess K^+.

Inhibition of PPFase by Na^+ and Ca^{++} has been studied in relation to both substrates, namely PEPA and ADP. Each point on a graph required four individual determinations to be run, viz. a control and the assay containing the inhibitor were run in duplicate. Per cent inhibitions quoted in the tables to be found in the appendix refer to the percentage by which the velocity of a control is reduced by adding a given amount of inhibiting ion. Twenty-minute preincubation periods have been used with both inhibiting ions.

Although an extremely weak activator of PPFase in the absence of K^+, Na^+ is a moderately powerful inhibitor of this enzyme in the presence of K^+ or some other univalent activating cation. Li^+ has been found to be a much more potent inhibitor of PPFase than Na^+ (47).

An analysis of Na^+ inhibition with varying concentrations of PEPA (Fig. 19, Table A-14) suggests that a significant amount of the inhibition by Na^+ may be attributed to coupling of the inhibiting ion with the substrate to form a complex which cannot enter into reaction with the enzyme. That Na^+ exerts its greatest effect by coupling with PEPA when present in relatively high concentrations is evidenced by the fact that increasing the (PEPA) from 0.00020 M to 0.00120 M reduces Na^+ inhibition from 81.9% to 30.9% at 0.15 M Na^+, but only reduces the inhibition by 0.05 M Na^+ from 14.7% to 9.2%.

Na^+ inhibition therefore resembles inhibition by excess K^+, except that inhibition by the former is more marked and is measurable at concentrations as low as 0.03 M, whereas inhibition by K^+ is evident at concentrations

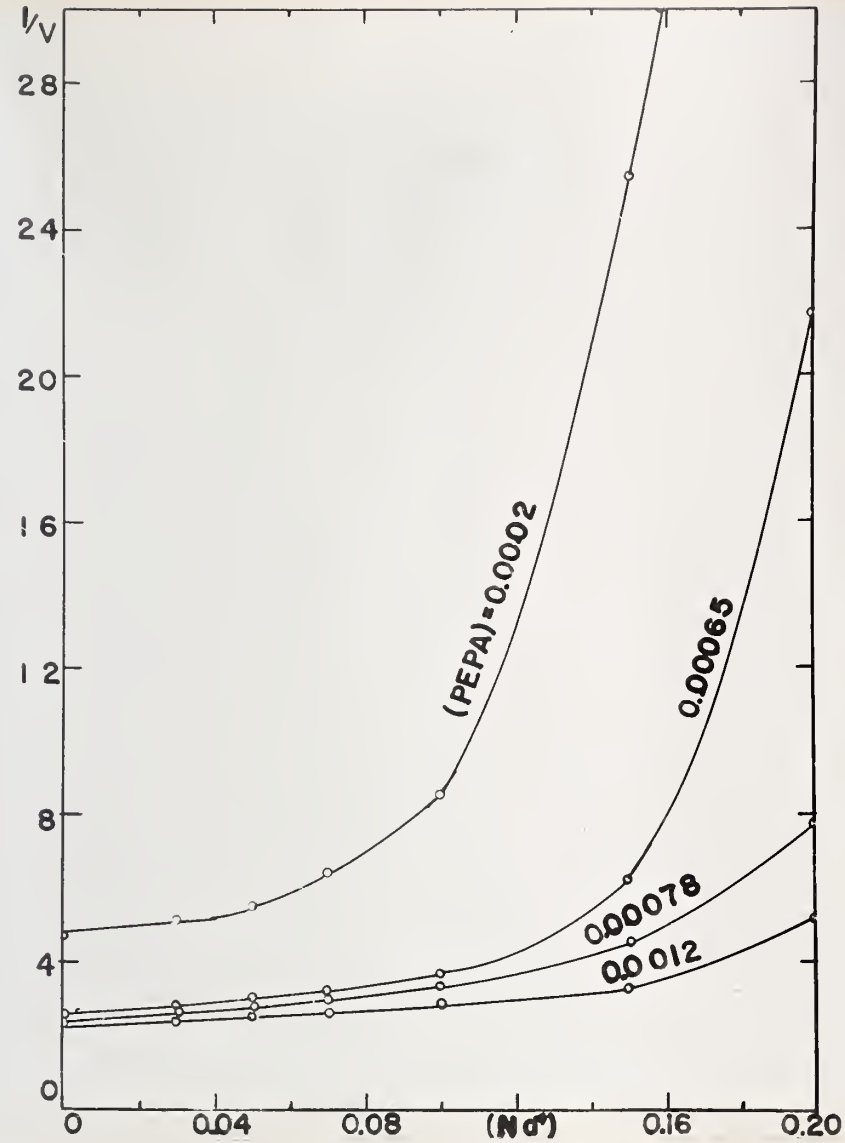


Fig. 19. Na^+ Inhibition of PPFase with Varying (PEPA) L-B plot of 1/V vs. (Na^+)

in excess of 0.075 M only. Of course the possibility that inhibition at low concentrations of Na^+ is due to interference with K^+ activation cannot be disregarded at this stage.

The binding of Na^+ by PEPA seems quite possible in the light of recent investigations which used the technique of Nuclear Magnetic Resonance, where binding of Na^+ with pyruvate, citrate, and lactate was observed in 0.01 N solutions of Na^+ (114). Because of its greater inhibiting power it would be expected that Li^+ would form stronger complexes with PEPA than does Na^+. A survey of the properties of the alkali metals reveals that a correlation can be made between their inhibiting capacity in the PPFase system and their position in the electronegativity scale of the elements. The following values of electronegativity for metal ions of importance in the PPFase system have been taken from lists compiled by Pauling (124) and Coryell (125):

Co = 1.7	Li = 1.0
Mn = 1.4	Na = 0.9
Mg = 1.2	K = 0.8
Ca = 1.0	Rb = 0.8

Coryell notes that there is a strong correlation between the electronegativity values of different metal atoms and the strength of complexes these metals form with a given type of ligand, irrespective of the nature of the complex forming ligand. This statement seems to complement the theory proposed.

The relation between K^+ activation and Na^+ and Ca^{++} inhibition of PPFase has been studied previously by Kachmar and Boyer (47). Preliminary results on Na^+ and Li^+ inhibition were interpreted by these workers on the

basis that Na^+ and Li^+ are antagonistic to K^+. From their experiments on Ca^{++}-K^+ antagonisms they conclude that the inhibition of PPFase by Ca^{++} appears complex and that although Ca^{++} exerts an inhibitory effect which for the most part cannot be removed by increasing the (K^+), there is a portion at low (Ca^{++}) which appears to be of the "apparent competitive" type and which can be overcome by increasing the (K^+).

A L-B plot of 1/V vs. $1/(K^+)$ (Fig. 20, Table A-15) suggests that the inhibition by Ca^{++} with respect to K^+ is noncompetitive. It may be of significance that an increase in (K^+) from 0.01 M to 0.075 M increases the percent inhibition by an absolute value of 6.9% with a (Ca^{++}) of 0.0005 M while at a (Ca^{++}) of 0.003 M the same increase in (K^+) results in an increase of 18%. Kachmar and Boyer, as noted previously, also found that K^+ could relieve Ca^{++} inhibition to some extent at lower (Ca^{++}).

The interaction between Na^+ and K^+ has also been studied (Fig. 21, Table A-16). The type of nonlinearity exhibited in this graph and subsequent graphs of the class 1/V vs. (Na^+) can be attributed to binding of (Na^+) by PEPA (cf. Fig. 19). Any modification of such plots will be due to the superimposition of effects produced by other variables in the system.

From this graph it is apparent that inhibition is much less at 0.02 M K^+ and high (Na^+) than at 0.050 M or 0.075 M K^+ and comparable (Na^+). In fact increasing the (K^+) from 0.050 to 0.075 M has little or no effect on Na^+ inhibition. The apparent lowering of Na^+ inhibition at low (K^+) can be explained on the basis that at such a (K^+) the rate-limiting effect of removal of PEPA through complex formation with Na^+ becomes less of a rate determining factor in view of the smaller amount of activated enzyme present; i.e. the average turnover rate of the enzyme molecules is effectively reduced,

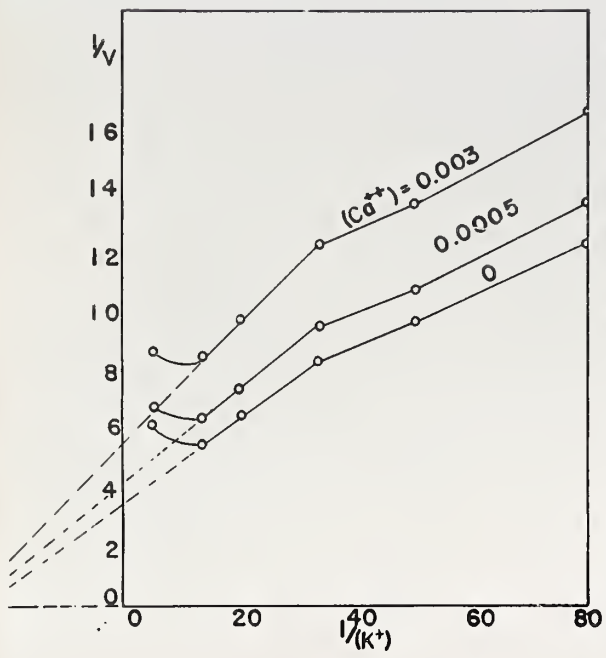


Fig. 20. Ca^{++} Inhibition of PPFase with Varying (K^+). L-B plot of 1/V vs. $1/(K^+)$

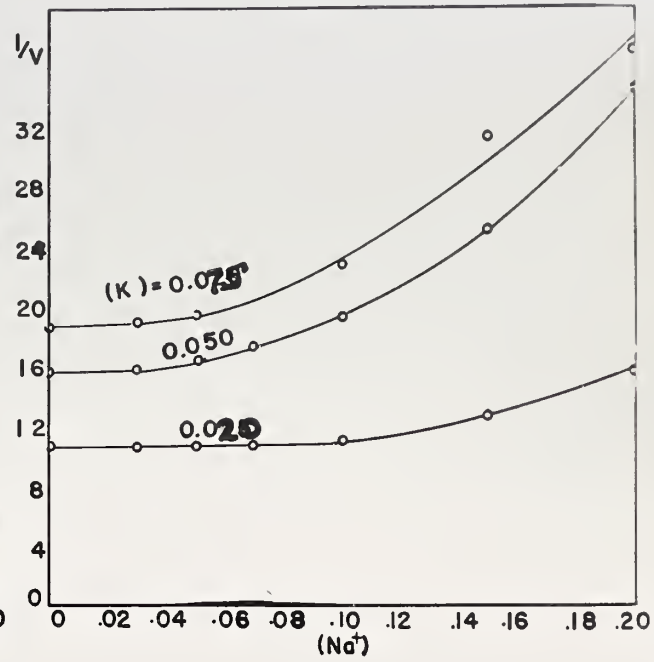


Fig. 21. Na^+ Inhibition of PPFase with Varying (K^+)* L-B plot of 1/V vs. (Na^+)

*The molarities of K^+ should read from top to bottom 0.075, 0.050 and 0.020 respectively.

and K^+ activation, not (PEPA), has become the main rate-limiting factor. The differences are quite marked. At 0.07 M Na^+ the per cent inhibitions at 0.02 M and 0.075 M K^+ are 0 and 15% while at 0.15 M Na^+ they are 17% and 40% respectively. The differences in per cent inhibition when 0.05 M K^+ and 0.075 M K^+ are used are not significant since the former (K^+) gives velocities very close to those of the optimum concentrations considered to be 0.075 M. In this study a special reagent grade NaCl "for biological work" (Merck) was used exclusively and this apparently gave slightly lower percent inhibition values than ordinary reagent grade NaCl. However the results are of relative importance and this fact does not reduce their significance. All evidence seems to indicate that Na^+ inhibition of PPFase is noncompetitive with respect to K^+.

The effect of Mg^{++} on Na^+ inhibition (Fig. 22, Table A-17) is not immediately open to interpretation. An extrapolation of the 1/V vs. $1/(Mg^{++})$ plots at three different (Na^+) suggests noncompetitive inhibition by Na^+ with respect to Mg^{++}. It is apparent from Table A-17, however, that the situation is not quite so simple, since increasing the (Mg^{++}) from 0.002 M to 0.018 M decreases Na^+ inhibition by an absolute amount of approximately 15% at all three concentrations of Na^+. Furthermore, by increasing the (Mg^{++}) from 0.001 M to 0.0014 M at 0.05 M Na^+ it is possible to decrease Na^+ inhibition from 25% to zero. Since the dissociation constant of the Mg - PEPA complex is relatively low, i.e. 10^{-2} M. at an ionic strength of 0.2 (122) and since the indications are that Na^+ exerts its inhibitory action by complex formation with PEPA, it is possible that Mg^{++} may complex at some region of the PEPA molecule whereby it either: (1) replaces Na^+, or (2) more likely is bound to PEPA at some point other than that at which the Na^+ is bound and

decreases the affinity of PEPA for the latter through electron withdrawal. It must be assumed in both cases that complexing of Mg^{++} with PEPA, which must also occur in the absence of any inhibitors, cannot be rate-limiting. At the same time it is doubtful whether such complexing of Mg^{++} with the substrate is a necessary prerequisite to coupling with the enzyme since the Mg-E dissociation constant is much less, yet graphical analysis indicates that this is the rate-determining dissociation in Mg^{++} activation.

A study of Na^{+} inhibition with respect to ADP as the variable substrate (Fig. 23, Table A-18) suggests that the complexing of Na^{+} with ADP has very little rate-limiting effect. Any reduction in Na^{+} inhibition at lower (Na^{+}) upon increasing (ADP) is no doubt due to a lowering of the thermodynamic activity of Na^{+} through complex formation. Comparison of this graph with a similar type of graph showing the effect of increasing the (Mg^{++}) on Na^{+} inhibition (Fig. 24, Table A-19) makes this interpretation more apparent.

Calcium inhibition has been studied over a range of (Ca^{++}) at several different PEPA concentrations (Fig. 25, Table A-20). The results are very difficult to interpret but it is obvious that Ca^{++} is a very potent inhibitor of PPFase; 0.0002 M is capable of producing 12.7% inhibition at 0.00078 M PEPA. There is an apparent increase in inhibition as the substrate concentration is increased to its optimum, which indicates that the inhibition is noncompetitive in nature. The family of lines approach linearity only at higher (PEPA).

The nature of Ca^{++} inhibition becomes quite obvious when its relation to Mg^{++} activation is studied. A plot of 1/V vs. $1/(Mg^{++})$ with varying Ca^{++} concentrations (Fig. 26, Table A-21) provides an example of perfect competitive

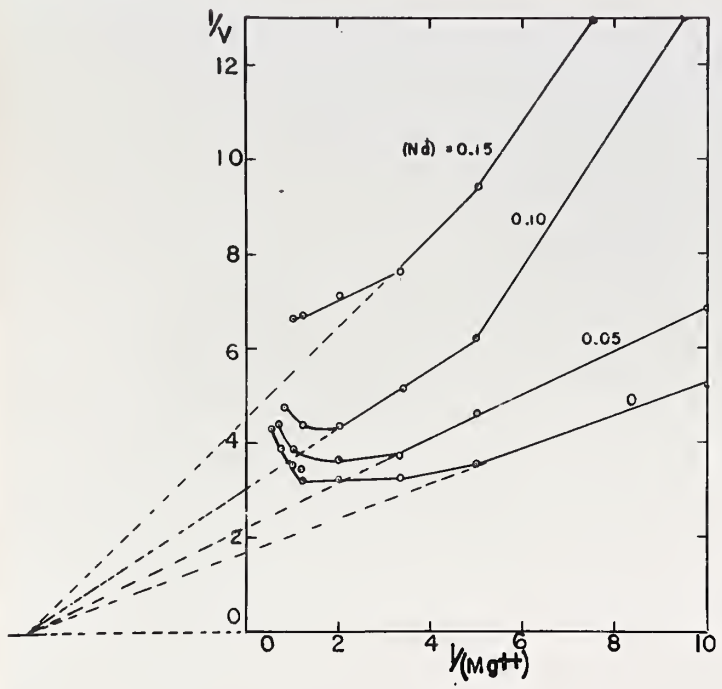


Fig. 22. Na^+ Inhibition of PPFase with Varying (Mg^{++})
L-B plot of 1/V vs. $0.1/(Mg^{++})$

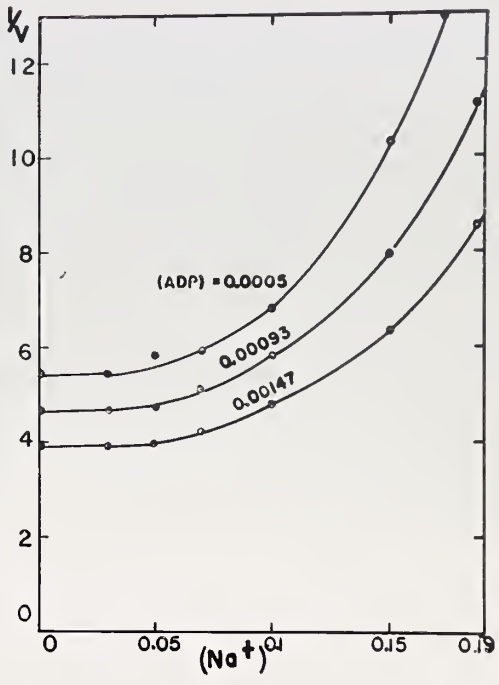


Fig. 23. Na^+ Inhibition of PPFase with Varying(ADP).
Plot of 1/V vs. Na^+

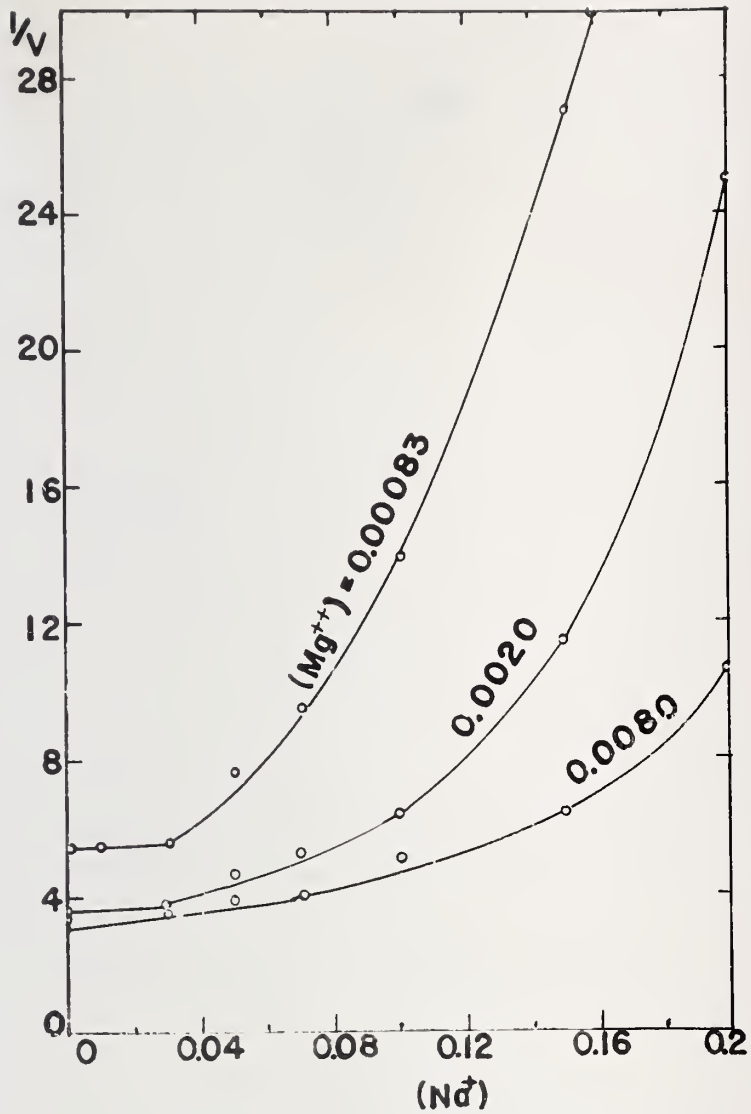


Fig. 24. Na^+ Inhibition of PPFase with Varying (Mg^{++}) Plot of 1/V vs. (Na^+)

inhibition; i.e. the maximum velocity is unaltered, and the slopes of the 1/V vs. $1/(Mg^{++})$ plots are directly proportional to the inhibitor concentration. This indicates that Ca^{++} must have only one main point of action on the PPFase system and that it lowers the effective enzyme concentration by directly interfering with Mg^{++} activation.

Figure 27 (Table A-22) shows the effect that varying the (ADP) has upon Ca^{++} inhibition.

Since the scale used on this graph is much smaller, and since the optimum (PEPA) was used, deviations from linearity are less apparent. The inhibition however appears to be competitive since the family of lines when extrapolated have a common intercept. Such a situation is difficult to reconcile with results already presented. The following argument offers an alternative explanation.

Since Ca^{++} shows true competitive inhibition with respect to Mg^{++} it is difficult to find a mechanism whereby a substrate such as ADP could relieve such inhibition, other than by reducing the free (Ca^{++}) through complex formation. Furthermore evidence has already been presented which indicates that Mg^{++} and ADP combine with the enzyme independently of one another. Another possible explanation is that binding of ADP by the enzyme may in some manner reduce the binding of Ca^{++} but not that of Mg^{++}. The "apparent competitive inhibition as described by Segal (101) does not offer a plausible explanation, since Ca^{++} inhibition appears to be an all or nothing affair. It is obvious that more information is necessary before any plausible mechanism for the reduction of Ca^{++} inhibition by increasing the (ADP) can be offered.

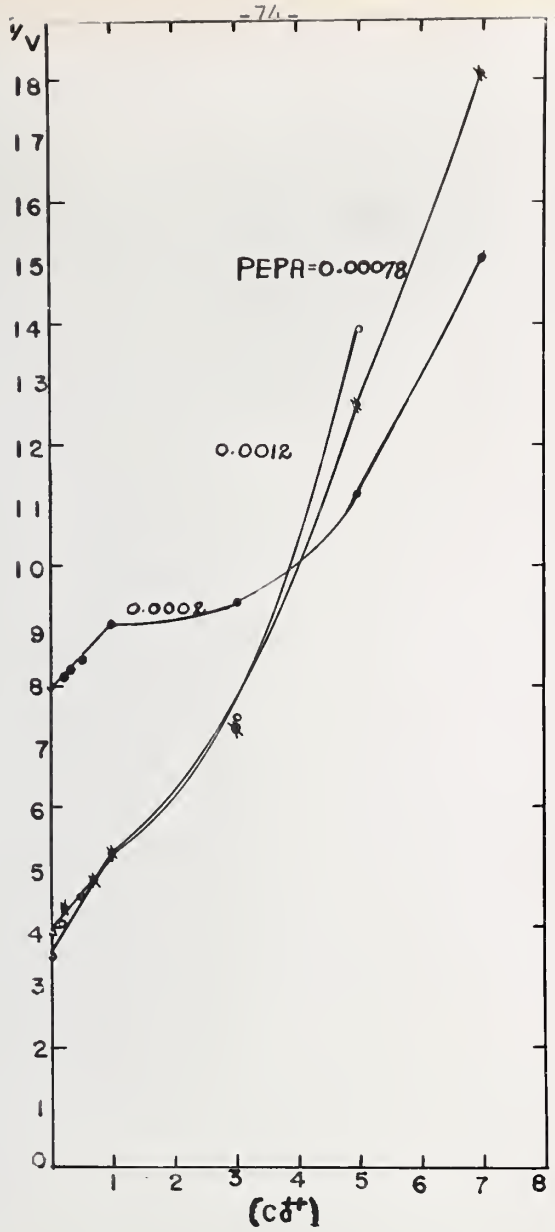


Fig. 25. Ca^{++} Inhibition of PPFase with Varying (PEPA) Plot of 1/V vs. (Ca^{++}) x 10^3

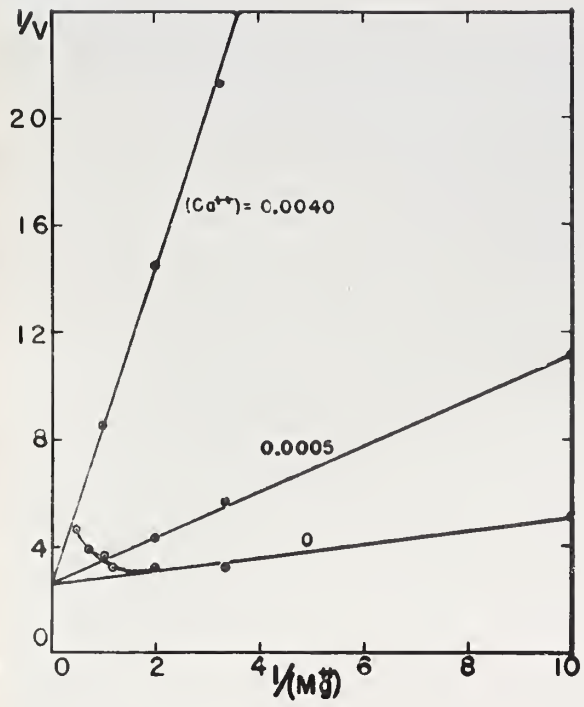


Fig. 26. Ca^{++} Inhibition of PPFase with Varying (Mg^{++})
L-B Plot of 1/V vs. $0.1/(Mg^{++})$

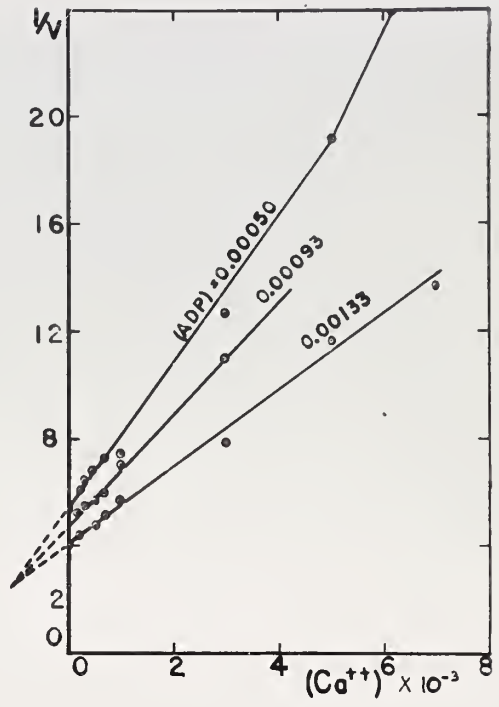


Fig.27. Ca^{++} Inhibition of PPFase with Varying (ADP)
Plot of 1/V vs. $(Ca^{++}) \times 10^{3}$

4. THE EFFECT OF pH

The shape of the V vs. pH curve (Fig. 28, Table A-23) only approaches the perfect bell-shaped curve often encountered in simple enzyme systems. The optimum pH is very close to 7.5. Although tris-maleate buffer was substituted for tris buffer in several of the determinations below pH 6.9, a comparison of activities with tris-maleate and tris buffers at pH 6.90 showed that both buffers gave identical results, i.e. PPFase activity appeared to be uninfluenced by the increased ionic strength and the presence of maleate.

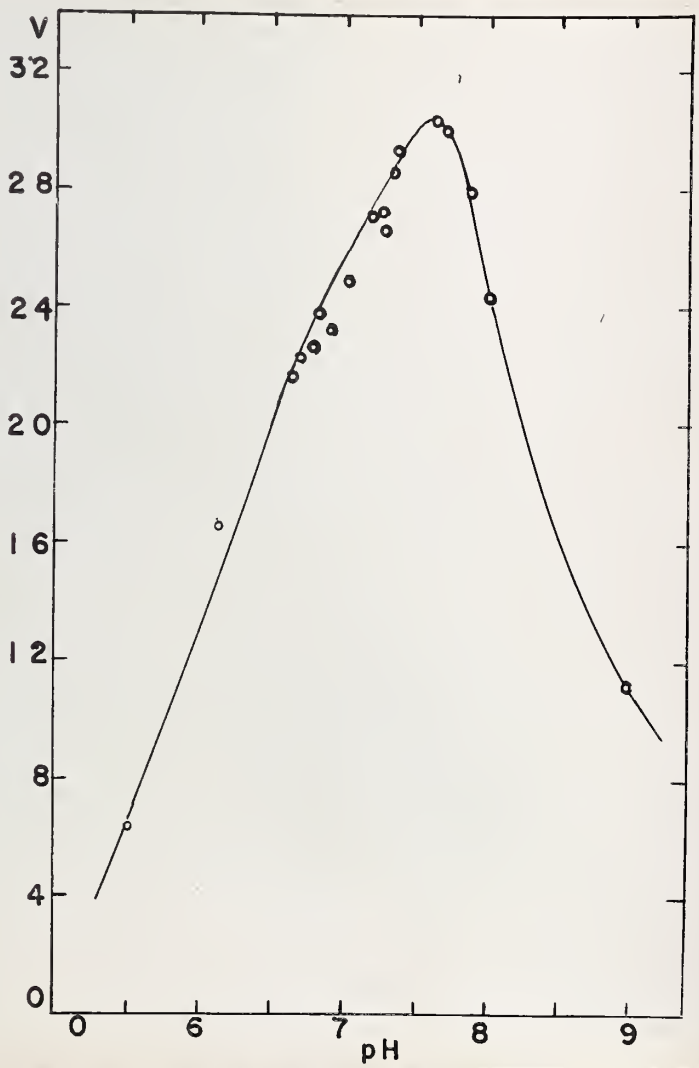


Fig. 28. Influence of pH on PPFase Activity. Plot of V vs. pH.

A few of the more recent studies on the influence of pH on enzymatic reactions have been performed on the metal-ion requiring enzymes, enolase (106) and arginase (107). The interpretation of pH effects in a system as complicated as the PPFase system requires that a relatively large number of variables need be considered, or that certain simplifying assumptions be made. The analysis can be simplified considerably by assuming that the change in (H^+) occurring with changes in pH has very little effect on the ionization of the substrate within the pH range investigated. This is the usual assumption which is found in the literature.

The Michaelis Constants obtained from L-B plots, for K^+ Mg^{++} and PEPA at various pH values are listed below.

TABLE 17
The Influence of pH on the Michaelis Constant for K^+
(Cf. Fig. 29, Table A-24)

pH	Δ pH	K_a	Δ K_a	$\frac{\Delta K_a}{\Delta pH}$
6.01	--	0.100	--	
6.86	+0.85	0.062	-0.038	4.5
7.36	+0.50	0.035	-0.027	5.4
8.11	+0.75	0.033	-0.002	0.3
9.01	+0.90	0.0250	-0.008	0.9

TABLE 18
The Influence of pH on the Michaelis Constant for Mg^{++}
(Cf. Fig. 30, Table A-25)

pH	Δ pH	K_a	ΔK_a	$\frac{\Delta K_a}{\Delta pH}$
6.16		0.00143		
6.70	+0.54	0.00154	+0.00011	21.0
7.46	+0.76	0.00125	-0.00029	3.8
8.04	+0.58	0.00083	-0.00042	7.2
8.66	+0.62	0.00063	-0.00020	3.2

TABLE 19
The Influence of pH on the Michaelis Constant for PEPA
(Cf. Fig. 31, Table A-26)

pH	Δ pH	K_a	ΔK_a	$\frac{\Delta K_a}{\Delta pH}$
6.75	--	0.00022		
7.46	0.71	0.00091	+0.00069	9.7
7.96	0.50	0.0050	+0.00409	32.0

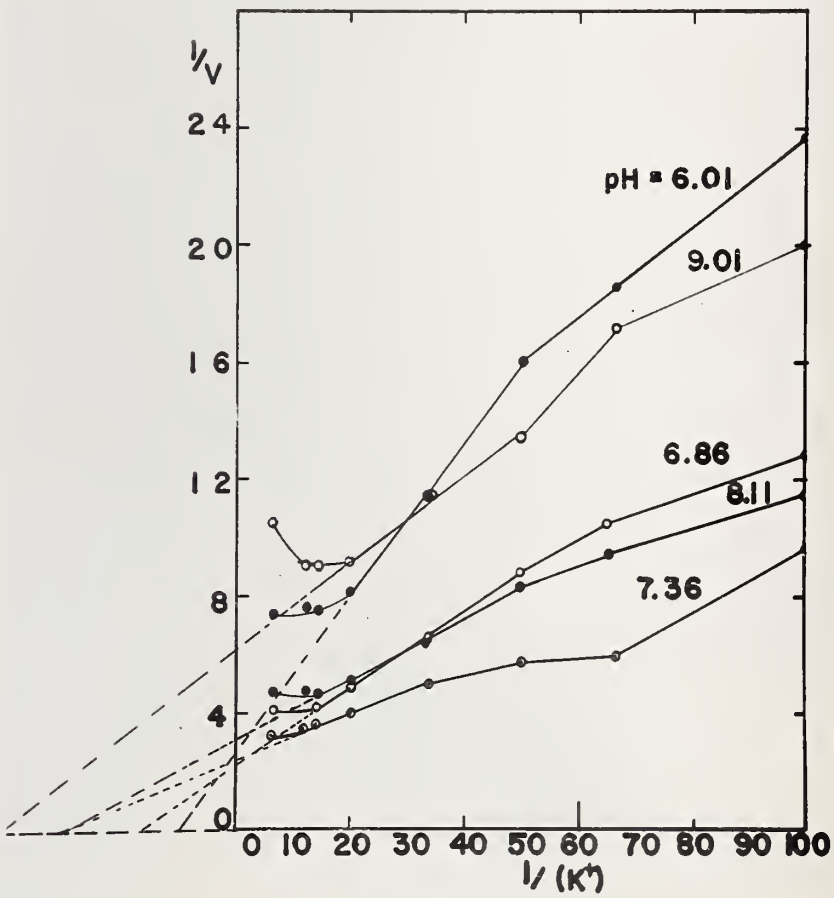


Fig. 29. K^+ Activation of PPFase with Varying pH
L-B plots for 1/V vs. $1/(K^+)$

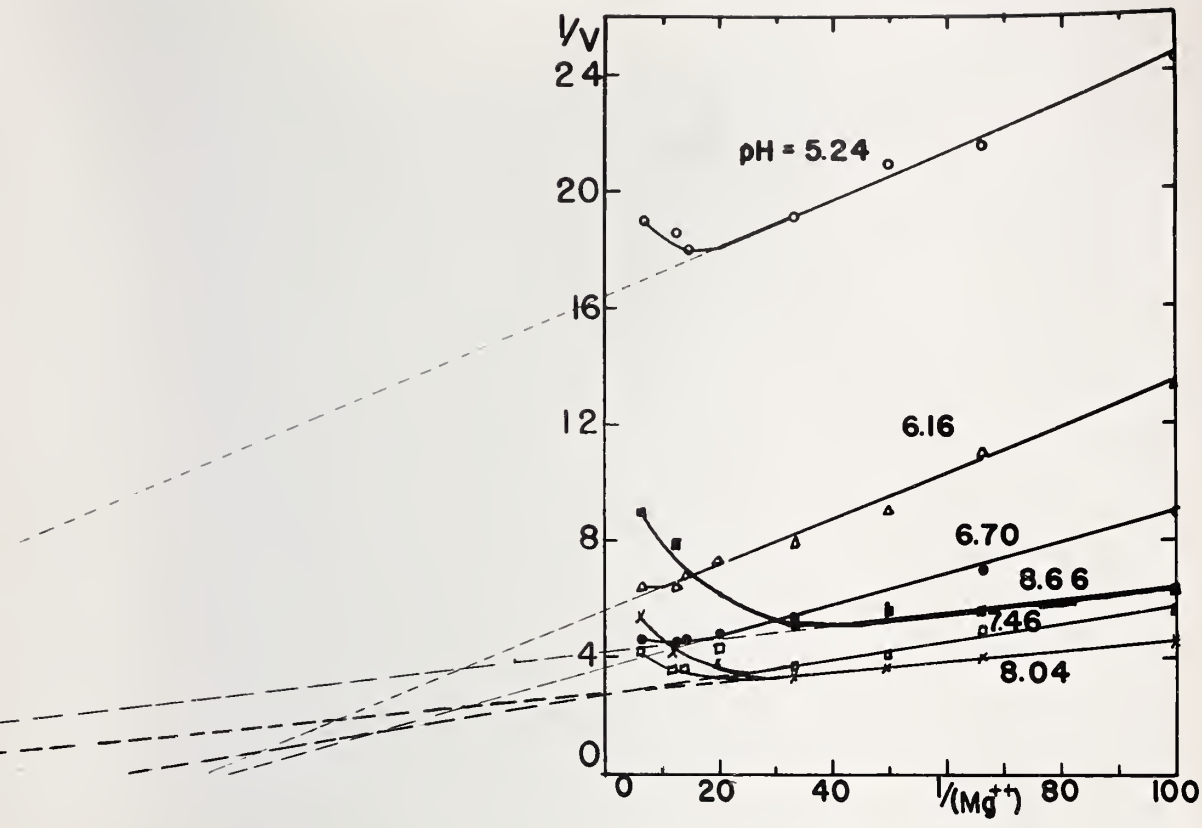


Fig. 30. Mg^{++} Activation of PPFase with Varying pH
L-B plots for 1/V vs. $1/(Mg^{++})$

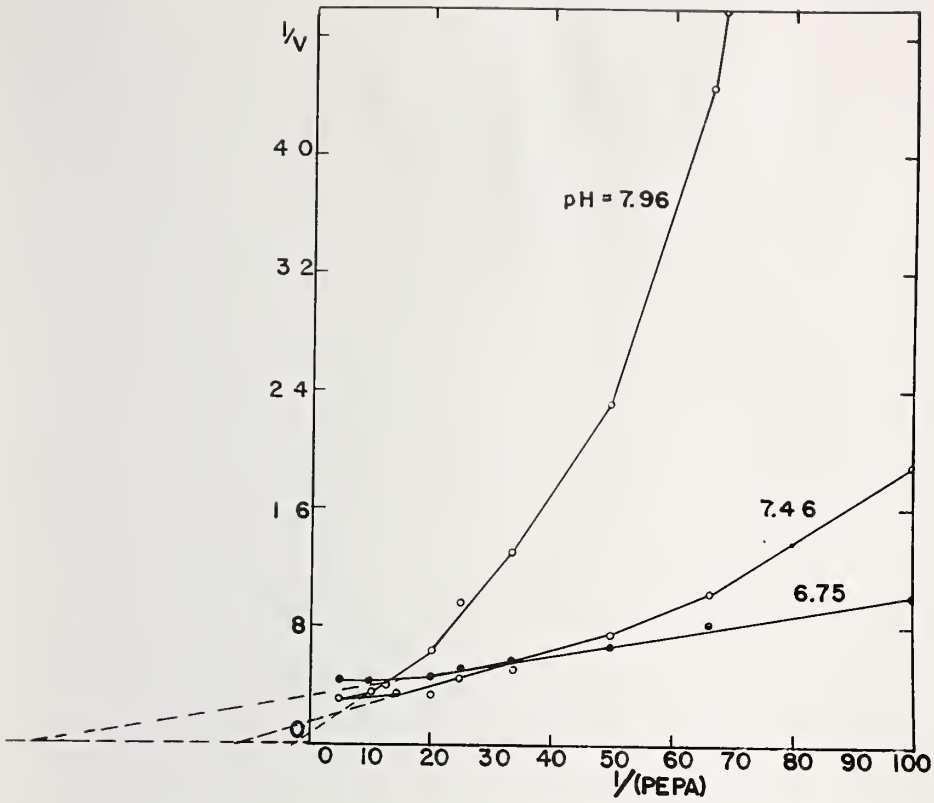


Fig. 31. Variation of the Michaelis Constant for PEPA with Varying pH. L-B plots for 1/V vs. 1/(PEPA).

There is a general drift in the Michaelis constants of all three of PEPA, Mg^{++} and K^{+}, the change in the Michaelis constant for PEPA being in a direction opposite to that for K^{+} and Mg^{++}. Although extrapolation of the L-B plots is difficult due to the nonlinearity at lower concentrations, and although the change in the Michaelis constant is not uniform with a change in pH in many cases, the general trend is quite evident.

The fact that the Michaelis constant decreases as the pH increases in the case of the activating ions is in accordance with the theory that the ionic group to which Mg^{++} or K^{+} is bound becomes more highly ionized as the pH is raised, thus increasing its affinity for the metal ions. On the

other hand an increasing pH would presumably raise the number of hydroxyl ions in the vicinity of a positively charged group in the active locus of the enzyme, thereby interfering with the coupling of the negatively charged carboxyl or negatively charged phosphate group of PEPA.

5. Discussion

Several questions arose from the kinetic analysis of cation interactions in the PPFase system for which no adequate answers can be given at present. The reason why a univalent cation such as K^+ is selectively bound at a certain region of the enzyme molecule whereas other univalent cations such as Na^+ and Li^+, and divalent cations such as Mg^{++} are not is certainly a point for conjecture. It does appear that Ca^{++} may have a slight competitive effect with respect to K^+ activation. The plot of $1/V$ vs. $1/(Mg^{++})$ with varying Ca^{++} concentration (Fig. 26) by its very nature conceals small inhibiting influences at other centers, since the (Ca^{++}) concentration is held constant, and the changes in the slopes of the lines resulting from such inhibition would be slight.

Just where the Na^+ or Li^+ is bound to the PEPA molecule is not known. Nuclear magnetic resonance data (114) suggest that greatest binding with Na^+ occurs with those compounds which have a hydroxyl or keto group in the α or β-position to a carboxyl group. Whether such coupling would actually occur with a keto group α to the carboxyl is open to conjecture, since reduction of such a group in pyruvic acid by LDH is uninfluenced by the presence of most cations including Na^+. PEPA, of course, has a phosphate group in an ester linkage at the α position and Na^+ is known to couple with the phosphate groups in ADP and ATP (111a).

Malmstrom (116) has found some evidence that divalent activating cations act as bridges from the enzyme to phosphoglyceric acid (PGA), and PEPA in the forward and reverse reactions respectively. Mg^{++} is known to bind with PEPA, and the absorption of PEPA at 240 mμ which is a function of the (Mg^{++}) has been utilized by Warburg and Christian (126) to study the extent of binding of Mg^{++} by PEPA. Comparisons of binding of Mg^{++} by both PEPA and PGA show that it is bound to the same extent by both substrates (116). Since data given by Martell and Calvin (110) indicate that the pK for Mg-lactate complex is 0.93 at an ionic strength of 0.2 whereas that for the Mg-PEPA complex is 2 under the same conditions (122), indicates that the phosphate group of PEPA must be involved in the complex formation. While there is no evidence that in order to form an active complex the activating ion must enter into direct union with both the PPFase enzyme molecule and PEPA, there exists this possibility. Studies on the influence of pH on the Michaelis constants of both Mg^{++} and PEPA would suggest that in such a case PEPA must be bound at another site, in addition to being bound to the enzyme by Mg^{++}.

No reason can be given for the apparent activation by Mg^{++} and inhibition by Ca^{++} of the PPFase enzyme. Although Mg-Ca antagonism is recognized as a physiological phenomenon there are exceptions. For instance, although Mg^{++} activates and Ca^{++} inhibits arginine phosphokinase, creatine phosphokinase is activated by both Mg^{++} and Ca^{++} (117). The mechanism through which ADP reduces Ca^{++} inhibition is also a problem for the future.

Boyer (50) has concluded from a study of the incorporation of pyruvate-2-C^{14} into PEPA, that the reaction catalyzed by PPFase does not proceed by formation of an enzyme-phosphate intermediate formed from the

enzyme and PEPA independently from the combination with ADP. Boyer and Harrison (127) note that in phosphate transfer from PEPA to ADP there is no exchange of oxygen phosphate with that of water during the transfer. The oxygen molecule of the acceptor molecule (ADP) is visualized as attaching and forming a bond with, the positively charged phosphorus in PEPA, resulting in cleavage of the O-P bond in the C-O-P linkage of the donor. It is conceivable that increasing the pH will reduce the positive charge on the phosphorus of PEPA, and could account in part for the reduction in velocity at higher pH's.

As noted previously, the phosphate acceptor for PPFase does not necessarily have to include the adenine moiety since other phosphate acceptors - e.g. deoxy ADP - can also function. This may indicate that direct transfer of phosphate occurs from the PEPA-PPFase intermediate to ADP.

Although there is no immediately apparent connection between the cation interactions which have been elucidated in the PPFase system and cation transport or general physiological cation antagonisms a few implications are apparent. In order that Na^+ ions exert any inhibitory function on the PPFase system, it must exert its action through coupling with PEPA and not by direct action upon the PPFase enzyme. The (PEPA) thus becomes an important limiting factor. On the other hand Ca^{++} must exert its action through direct combination with the enzyme.

Martin (64) in discussing the many examples of *in vivo* and *in vitro* ion antagonisms has also recorded from the literature a few which exhibit antagonisms between cations important in the PPFase system. Toxicity due to injection of K^+ or NH_4^+ salts into guinea pigs, for instance, can be

reduced by administration of Na^+ salts. In addition, the ion ratios of Ca^+, K^+, Na^+, and H^+ alter many autonomic functions since excised heart, stomach, bladder, and uterus respond to an increase of the Ca/K ratio as to sympathetic, and an increase of the K/Ca ratio as to parasympathetic, stimulation. Prominent among the cation interactions more recently cited in the literature, are the Ca-Mg antagonisms in muscle (61) and the effect of Na^+ and K^+ on the respiratory rate of mitochondria (74).

Although there is at present no immediately apparent connection between cation interactions in the PPFase system and cation transport, subsequent research will undoubtedly prove or disprove the theory that PPFase is related to the mechanisms controlling active transport. These findings and others of a similar nature may be of value in the elucidation of biological antagonisms and in the elucidation of the mechanism of enzyme action itself.

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A P P E N D I X

TABLES A-1 to A-26

(Primary and Secondary Data)

TABLE A-1

K^+ Activation of PPFase with Varying (PEPA)
Primary and Secondary Data
for 1/V vs. $1/(K^+)$
(Fig. 4)

K^+ Conc. (M)	$1/(K^+)$	(PEPA) = 0.0002 M		(PEPA) = 0.0005 M		(PEPA) = 0.0012 M	
		V*	100/V	V	100/V	V	100/V
0.010	100	4.6	21.7	11.5	8.7		7.0
0.030	33.3	13.0	7.7	23.3	4.3	24.0	4.2
0.050	20.0	17.3	5.8	30.3	3.3	33.0	3.0
0.060	16.7	19.0	5.3	34.5	2.9	34.4	2.9
0.065	15.4	18.1	5.5	37.0	2.7	37.0	2.7
0.070	14.3	19.8	5.1	38.5	2.6	37.9	2.6
0.080	12.5	21.6	4.6	37.1	2.7	40.8	2.5
0.085	11.8	20.6	4.9	38.5	2.6	--	--
0.160	6.3	15.4	6.5	37.1	2.7	43.3	2.3
0.180	5.55	--	--	35.7	3.15	43.0	2.33

V* = units PPFase activity

TABLE A-2

Na^+ Activation of PPFase Data for V vs. (Na^+)
(Fig. 5)

Na^+ Conc. (M)	Activity (units)	Na^+ Conc. (M)	Activity (units)
--	2.60	0.0400	5.95
0.0025	2.62	0.0500	6.65
0.0050	3.22	0.0600	7.30
0.0100	4.16	0.0700	6.70
0.0200	4.89	0.1000	5.30
0.0300	5.89	0.2000	3.42

TABLE A-3

NH_4^+ Activation of PPFase with Varying (ADP)
Primary and Secondary Data for 1/V vs. $1/(NH_4^+)$
(Fig. 6)

NH_4^+ Conc. (M)	$1/(NH_4^+)$	(ADP) = 0.00050 M		(ADP) = 0.00093 M		(ADP) = 0.00147 M	
		V	100/V	V	100/V	V	100/V
0.01	100	6.9	14.5	9.4	13.5	9.0	11.1
0.02	50	10.2	9.8	12.5	8.0	14.6	6.85
0.03	33.3	12.9	7.8	15.9	6.3	18.9	5.3
0.04	25	14.6	6.85	17.7	5.65	21.9	4.6
0.05	20	16.3	6.15	20.4	4.9	23.4	4.28
0.06	16.7	16.6	6.0	21.4	4.7	24.6	4.06
0.07	14.3	18.3	5.5	21.6	4.64	25.4	3.95
0.08	12.5	18.6	5.4	21.6	4.64	23.8	4.20
0.09	11.1	18.3	5.5	21.6	4.64	--	--
0.10	10	--	--	21.0	4.76	23.0	4.35
0.12	8.4	18.3	5.5	20.4	4.90	--	--
0.16	6.3	--	--	19.0	5.28	21.4	4.68
0.20	5.0	17.4	5.75	18.1	5.51	20.5	4.88
0.30	3.3	--	--	15.3	6.55	15.3	6.55

TABLE A-4
K^+ Activation of PPFase with Varying (ADP)
Primary and Secondary Data for 1/V vs. $1/(K^+)$
(Fig. 7)

K^+		ADP = 0.00030 M		ADP = 0.00050 M		ADP = 0.00067 M		ADP = 0.00093 M		ADP = 0.00133 M	
Conc. (M)	$1/K^+$	V	1/V	V	1/V	V	1/V	V	1/V	V	1/V
0.010	100	5.8	17.3	7.2	13.9	6.5	15.4	9.2	10.9	10.7	9.35
0.020	50	10.4	9.6	10.5	9.5	11.5	8.7	12.6	7.95	13.8	7.25
0.030	33.3	12.0	8.3	13.3	8.12	13.5	7.4	15.0	6.65	16.3	6.15
0.050	20	12.5	6.8	15.6	6.4	17.2	5.8	19.8	5.05	20.6	4.85
0.075	13.4	14.7	5.9	18.6	5.4	19.7	5.1	21.7	4.60	24.4	4.10
0.100	10	15.2	6.6	18.6	5.4	19.7	5.1	22.2	4.50	23.9	4.20
0.160	6.3	--	--	18.0	5.55	18.4	5.4	--	--	23.1	4.31
0.250	4.0	--	--	16.5	6.05	--	--	15.8	6.31	16.6	6.02

TABLE A-5
Mg^{++} Activation of PPFase with Varying (ADP)
Primary and Secondary Data for 1/V vs. $1/(Mg^{++})$
(Fig. 8)

Mg^{++} Conc. (M)	$0.1/(Mg^{++})$	ADP = 0.00054 M		ADP = 0.00070 M		ADP = 0.00093 M		ADP = 0.00147 M	
		V	1/V	V	1/V	V	1/V	V	1/V
0.0010	100	11.0	9.1	10.6	9.5	13.6	7.35	14.2	7.1
0.0015	66.5	12.7	7.9**	--	--	--	--	--	--
0.0020	50	14.5	6.9	16.0	6.3	18.0	5.6	21.0	4.76
0.0030	33.3	16.8	6.0	19.0	5.3	20.4	4.9	24.6	4.05
0.0040	25	17.3	5.8	--	5.2	21.0	4.76	25.0	4.00
0.0050	20	17.9	5.6	19.4	5.2	21.6	4.63	25.4	3.94
0.0060	14.7	18.3	5.5	19.4	5.0	21.0	4.76	25.4	3.94
0.0080	12.5	18.6	5.4	20.0	5.3	21.6	4.63	25.4	3.94
0.0090	11.1	--	--	18.8	5.9	--	--		
0.0100	10	17.3	5.8	--	--	21.1	4.71	22.9	4.38
0.0140	7.2	16.4	6.1	--	--	16.7	6.00	19.8	5.05

**Not indicated on graph

TABLE A-6

Mn^{++} Activation of PPFase with Varying (ADP)
Primary and Secondary Data for 1/V vs. $1/(Mn^{++})$
(Fig. 9)

Mn^{++} Conc. (M)	$0.1/(Mn^{++})$	(ADP) = 0.00050 M		(ADP) = 0.00093 M		(ADP) = 0.00147 M	
		V	1/V	V	1/V	V	1/V
0.0010	100	6.0	16.8	6.0	16.7	7.1	14.1
0.0015	66.5	8.1	12.3	9.6	10.4	8.1	12.5
0.0020	50	10	10.0	--	--	9.9	10.1
0.0025	40	--	--	12.1	8.3	--	--
0.0030	33.3	12.7	7.9	14.4	7.1	15.6	6.4
0.0040	25	13.7	7.3	16.9	5.9	18.2	5.5
0.0050	20	16.0	6.3	18.6	5.4	22.2	4.5
0.0060	16.7	16.9	5.95	20.5	4.9	22.5	4.45
0.0070	14.3	17.7	5.65	--	--	24.0	4.15
0.0080	12.5	18.6	5.4	21.6	4.6	25.4	3.95
0.0090	11.1	18.6	5.4	--	--	--	--
0.160	6.25	17.1	5.85	--	--	20.0	5.00

Mg^{++} Activation of PPFase with Varying (PEPA)
Primary and Secondary Data for 1/V vs. $1/(Mg^{++})$
(Fig. 10)

Mg^{++} Conc. (M)	$0.1/(Mg^{++})$	(PEPA) = 0.00020 M		(PEPA) = 0.00050 M		(PEPA) = 0.00078 M	
		V	100/V	V	100/V	V	100/V
0.001	100	9.2	10.8	19.2	5.2	23.8	4.2
0.002	50	14.5	6.9	24.8	4.1	31.8	3.2
0.003	33.3	16.4	6.1	29.7	3.4	33.3	3.0
0.005	20	16.3	6.2	32.0	3.1	34.5	2.9
0.008	12.5	13.0	7.7	31.5	3.2	34.5	2.9
0.016	6.25	6.9	14.4	19.6	5.1	28.2	3.6

TABLE A-8
Mg^{++} Activation of PPFase
Primary and Secondary Data for 1/V vs. 1/(PEPA)
(Fig. 11)

PEPA Conc. (M)	0.01/(PEPA)	(Mg^{++}) = 0.0015		(Mg^{++}) = 0.0045		(Mg^{++}) = 0.0080	
		V	100/V	V	1/V	V	1/V
0.00014	70	10.8	9.3	9.4	10.6	7.7	13.0
0.00020	50	14.5	6.9	16.6	6.0	12.7	7.9
0.00030	33.3	19.6	5.1	24.0	4.2	19.2	5.2
0.00050	20	25.5	3.9	31.5	3.2	26.3	3.8
0.00070	14.3	27.5	3.7	32.7	3.1	33.3	3.0
0.00080	12.5	28.4	3.5	33.9	3.0	35.7	2.8
0.00140	7.3	28.4	3.5	36.0	2.8	35.7	2.8

TABLE A-9
Inhibition by (Mg^{++}) in Excess of Optimum with Varying (PEPA)
Primary and Secondary Data for 1/V vs. Molar Excess Mg^{++}
(Fig. 14)

(Mg^{++}) Total (M)	(Mg^{++}) Molar Excess*	(PEPA) = 0.00020			(PEPA) = 0.00040			(PEPA) = 0.00078		
		% I	V	1/V	% I	V	1/V	% I	V	1/V
0.0080	0.00	--	13.5	7.4	--	24.4	4.1	--	33.3	3.0
0.0085	0.0005	3.3	13.0	7.7	0	"	"	0	"	"
0.0095	0.0015	14.0	11.6	8.6	0	"	"	0	"	"
0.0105	0.0025	21.8	10.6	9.5	1.7	24.0	4.2	0	"	"
0.0120	0.0040	31.4	9.3	10.7	10.3	21.9	4.6	0	"	"
0.0140	0.0060	38.4	8.4	11.9	15.5	20.6	4.9	7.2	30.9	3.3
0.0160	0.0080	45.0	7.5	13.2	26.6	17.9	5.6	9.5	30.1	3.3
0.0180	0.0100	46.0	7.3	13.8	28.3	17.5	5.7	--	--	--
0.0200	0.0120	54.0	6.2	16.1	28.4	17.5	5.7	17.5	28.5	3.5

*(Mg^{++}) in excess of 0.0080 M

Inhibition by (Mg^{++}) in Excess of Optimum with Varying (ADP)
Primary and Secondary Data for 1/V vs. Molar Excess Mg^{++}
(Fig. 15)

(Mg^{++}) Total (M)	(Mg^{++}) Molar Excess*	(ADP) = 0.00040			(ADP) = 0.00067			(ADP) = 0.00147		
		% I	V	1/V	% I	V	1/V	% I	V	1/V
0.0080	0.00	--	30.4	3.3	--	33.3	3.00	--	43.0	2.33
0.0085	0.0005	0	"	"	0	"	"	0	"	"
0.0100	0.0020	0	"	"	0	"	"	0	"	"
0.0120	0.0040	10	27.4	3.67	0	"	"	0	"	"
0.0140	0.0060	13.7	26.3	3.80	7.2	30.9	3.25	0	"	"
0.0160	0.0080	13.7	26.3	3.80	9.5	30.1	3.32	--	--	--
0.0180	0.0100	16.6	25.3	3.95	--	--	--	--	--	--
0.2000	0.0120	22.8	23.5	4.25	17.5	28.5	3.50	18.0	35.3	2.85

*(Mg^{++}) in excess of 0.0080 M

TABLE A-11
Inhibition of PPFase by (K^+) in Excess of Optimum with Varying (PEPA)
Primary and Secondary Data for 1/V vs. Molar Excess of K^+
(Fig. 16)

(K^+) Total (M)	(K^+) Molar Excess*	(PEPA) = 0.00040			(PEPA) = 0.00040			(PEPA) = 0.00078		
		% I	V	1/V	% I	V	1/V	% I	V	1/V
0.075	0.00	--	13.5	7.4	--	24.4	4.1	--	33.3	3.00
0.080	0.005	0	"	"	0	"	"	0	"	"
0.090	0.015	0	"	"	0	"	"	0	"	"
0.100	0.025	0	"	"	0	"	"	0	"	"
0.120	0.045	--	--	--	--	--	--	--	--	--
0.140	0.065	8.3	12.4	8.0	3.1	23.6	4.3	0	33.3	3.00
0.160	0.085	20.0	10.9	9.2	8.7	22.3	4.5	--	--	--
0.180	0.105	24.0	10.3	9.7	20.5	19.4	5.2	0	33.3	3.00
0.200	0.125	45.5	7.4	13.5	28.0	17.6	5.7	11.5	29.1	3.42
0.25	0.175	--	--	--	47.5	12.8	7.8	24.0	25.3	3.95
0.300	0.225	56	6.0	16.6	66.0	8.4	11.9	46.0	16.2	6.20

*(K^+) in excess of 0.075 M

TABLE A-12
Inhibition of PPFase by (K^+) in Excess of Optimum with Varying (ADP)
Primary and Secondary Data for 1/V vs. Molar Excess of K^+
(Fig. 17)

(K^+) Total (M)	(K^+) Molar Excess*	(ADP) = 0.00040 M			(ADP) = 0.00067 M			(ADP) = 0.00147 M		
		% I	V	1/V	% I	V	1/V	% I	V	1/V
0.075	0.00	--	30.4	3.30	--	33.3	3.00	--	43.0	2.33
0.090	0.015	0	"	"	0			0	"	"
0.100	0.025	2.8	29.5	3.40	0			0	"	"
0.120	0.045	5.6	28.7	3.50	--	--	--	--	--	--
0.140	0.065	5.6	"	"	0	33.3	3.00	1.84	42.2	2.36
0.160	0.085	5.6	"	"	--	--	--	1.84	"	"
0.180	0.105	12.5	26.6	3.75	0	33.3	3.00	7.80	39.7	2.50
0.200	0.125	12.5	"	"	11.5	29.1	3.42	12.20	37.7	2.65
0.250	0.175	18.0	24.9	4.00	24.0	25.3	3.95	22.0	33.5	3.00

* = (K^+) in excess of 0.075 M

TABLE A-13
Effect of Mn^{++} on Mg^{++} Activation of PPFase
Primary and Secondary Data for 1/V vs. 1/(PEPA)
(Fig. 18)

PEPA Conc. (M)	0.01/(PEPA)	(Mn^{++}) = 0.000		(Mn++) = 0.005 M		(Mn++) = 0.008 M	
		V	1/V	V	1/V	V	1/V
0.0001	100	6.4	15.6	7.2	13.9	5.65	17.7
0.0002	50	9.7	10.3	8.2	12.2	6.1	16.4
0.0004	25	13.0	7.7	9.3	10.8	6.4	15.8
0.0006	16.7	14.0	7.2	8.8	11.4	6.6	15.1
0.00078	12.8	14.5	6.9	8.8	11.4	6.8	14.7
0.0010	10.0	17.1	5.9	9.3	10.8	--	--
0.0016	6.3	17.1	5.9	9.3	10.8	6.8	14.7

TABLE A-14
Na^+ Inhibition of PPFase with Varying (PEPA)
Primary and Secondary Data for 1/V vs. (Na^+)
(Fig. 19)

Na^+	(PEPA) = 0.00020 M			(PEPA) = 0.00065 M			(PEPA) = 0.00078 M			(PEPA) = 0.00120 M		
Conc. (M)	% I*	V**	100/V	% I	V	100/V	% I	V	100/V	% I	V	100/V
0.0	--	21.3	4.7	--	38.4	2.60	--	43.4	2.30	--	43.4	2.30
0.03	9.29	19.3	5.2	7.0	35.7	2.80	15.0	36.9	2.70	2.8	42.3	2.36
0.05	14.7	18.2	5.5	14.0	33.01	3.01	18.0	35.6	2.80	9.2	39.4	2.54
0.07	27.3	15.5	6.45	18.5	31.3	3.20	22.0	33.9	2.96	11.6	38.4	2.60
0.10	45.0	11.7	8.55	28.9	27.3	3.66	31.5	29.8	3.36	20.0	34.7	2.87
0.15	81.9	3.9	25.5	58.0	16.1	6.20	48.6	22.3	4.50	30.9	30.0	3.33
0.20	91.5	1.9	52.5	88.0	4.6	21.70	70.0	13.0	7.69	55.5	19.4	5.18

*% I = % inhibition (average of duplicate determinations)
**V = units PPFase activity

TABLE A-15
Ca^{++} Inhibition of PPFase with Varying (K^+)
Primary and Secondary Data for 1/V vs. $1/(K^+)$
(Fig. 20)

K^+		$(Ca^{++}) = 0.00$		$(Ca^{++}) = 0.0005$ M			$(Ca^{++}) = 0.0030$ M		
Conc. (M)	$1/K^+$	V	1/V	% I	V	1/V	% I	V	1/V
0.010	100	7.2	13.9	10.3	6.5	15.33	35.0	5.3	18.8
0.020	50	10.5	9.5	11.6	9.4	10.6	42.0	7.4	13.5
0.030	33.3	12.4	8.1	12.6	10.6	9.4	46.5	8.3	12.1
0.050	20.0	15.6	6.4	14.5	13.7	7.3	50.0	10.4	9.6
0.075	13.4	18.5	5.4	17.2	15.9	6.3	53.0	12.0	8.3
0.200	5.0	--	--	7.6	15.4	6.5	--	--	--
0.250	4.0	16.6	6.1	--	--	--			

TABLE A-16
Na^+ Inhibition of PPFase with Varying (K^+)
Primary and Secondary Data for 1/V vs. (Na^+)**
(Fig. 21)

Na^+	$(K^+) = 0.020$ M			$(K^+) = 0.050$ M			$(K^+) = 0.075$ M		
Conc. (M)	% I	V	1/V	% I	V	1/V	% I	V	1/V
0.03	0	9.5	10.5	2.34	6.3	15.8	2.4	5.3	18.8
0.05	0	9.5	10.5	4.2	6.1	16.4	4.8	5.15	19.5
0.07	0	9.5	10.5	10.0	5.8	17.2	14.8	4.6	21.8
0.10	4.15	9.1	11.0	18.0	5.2	19.3	19.0	4.4	22.7
0.15	16.80	7.9	12.7	36.0	4.0	25.0	40.0	3.2	31.3
0.20	32.00	6.4	15.6	55	2.9	34.5	50.0	2.7	37.0

**The relative velocities at 0.02 M, 0.05 M, and 0.075 M K^+ with no Na^+ present should be 18.3, 27.0 and 32.2 respectively.

TABLE A-17
Na^+ Inhibition of PPFase with Varying (Na^+)
Primary and Secondary Data for 1/V vs. $1/(Mg^{++})$
(Fig. 22)

Mg^{++} Conc. (M)	$0.01/(Mg^{++})$	$(Na^+) \pm 0.00$ M		$(Na^+) = 0.05$ M			$(Na^+) = 0.10$ M			$(Na^+) = 0.15$ M		
		V	1/V	% I	V	1/V	% I	V	1/V	% I	V	1/V
0.0010	10.00	19.6	5.10	25.4	14.6	6.85	54.3	7.1	13.70	69.0	6.1	16.4
0.0020	5.00	28.0	3.55	22.8	21.6	4.64	42.4	16.1	6.21	61.5	10.6	9.4
0.0030	3.33	30.8	3.50	12.7	26.9	3.72	36.5	28.2	3.55	57.5	13.1	7.61
0.0050	2.00	31.3	3.20	12.2	27.5	3.64	26.7	22.9	4.38	55.2	14.0	7.15
0.0080	1.20	31.3	3.20	8.0	28.8	3.48	27.0	23.0	4.35	48.3	15.1	6.65
0.0099	1.01	--	--	--	--	--	--	--	--	46.5	15.4	6.50
0.010	1.00	28.6	3.50	1.0	25.7	3.88	--	--	--	--	--	--
0.012	0.81	--	--	--	--	--	20.3	21.3	4.70	--	--	--
0.014	0.72	25.6	3.90	0.0	25.6	3.91	--	--	--	--	--	--
0.018	0.56	23.2	4.30	--	--	--	--	--	--	--	--	--

TABLE A - 18
Na^+ Inhibition of PPFase with Varying (ADP)
Primary and Secondary Data for 1/V vs. (Na^+)
(Fig. 23)

Na^+	(ADP) = 0.0005 M			(ADP) = 0.00093 M			(ADP) = 0.00147 M		
Conc. (M)	% I	V	1/V	% I	V	1/V	% I	V	1/V
0.00	--	18.6	5.40	--	21.60	4.63	--	25.4	3.94
0.03	0	18.6	5.40	0	21.60	4.63	0	25.4	3.94
0.05	8.8	16.9	5.90	2.4	21.1	4.75	0	25.4	3.94
0.07	9.5	16.8	5.95	9.2	19.6	5.10	5.75	23.9	4.20
0.10	20.8	14.8	6.80	26.8	15.8	6.81	18.2	20.8	4.80
0.15	47.8	9.7	10.30	41.7	12.6	7.95	38.0	15.8	6.35
0.19	63.3	6.8	14.7	58.5	9.0	11.10	54.0	11.7	8.55

TABLE A-19
Na^+ Inhibition of PPFase with Varying (Mg^{++})
Primary and Secondary Data for 1/V vs. (Na^+)
(Fig. 24)

Na^+	(Mg^{++}) = 0.00083 M			(Mg^{++}) = 0.0020 M			(Mg^{++}) = 0.0080 M		
Conc. (M)	% I	V	1/V	% I	V	1/V	% I	V	1/V
0.03	1.4	18.1	5.5	3.9	26.9	3.7	15.0	26.8	3.72
0.05	28.4	13.2	7.6	16.5	23.4	4.3	18.0	25.9	3.86
0.07	43.0	10.5	9.5	30.8	19.3	5.2	22.0	24.6	4.06
0.10	61.0	7.2	13.9	44.0	15.7	6.4	31.0	21.8	4.57
0.15	85.6	3.7	27.0	68.5	8.8	11.4	48.6	16.2	6.20
0.20	92.0	1.5	66.5	85.0	4.0	25.0	70.0	9.5	10.50

TABLE A-20
Ca^{++} Inhibition of PPFase with Varying (PEPA)
Primary and Secondary Data for 1/V vs. (Ca^{++})
(Fig. 25)

(Ca^{++})	(PEPA) = 0.00020 M			(PEPA) = 0.00078 M			(PEPA) = 0.00120 M		
Conc. (M)	% I	V	100/V	% I	V	100/V	% I	V	100/V
0.00	--	12.50	8.00	--	28.60	3.5	--	28.60	3.50
0.0002	2.1	12.20	8.18	12.7	24.96	4.02	14.1	24.65	4.07
0.0003	2.6	12.18	8.24	18.1	23.42	4.28	18.8	23.22	4.30
0.0005	5.4	11.82	8.43	22.4	22.20	4.51	20.8	22.65	4.40
0.00070	--	--	--	27.4	20.79	4.80	24.8	21.49	4.65
0.0010	11.2	11.10	9.01	33.8	18.95	5.29	36.2	18.2	5.50
0.0030	15.0	10.62	9.20	52.5	13.6	7.35	54.0	13.2	7.60
0.0050	28.5	8.94	11.20	72.3	7.9	12.70	75.0	7.20	13.90
0.0070	47.0	6.62	15.10	81.0	5.5	18.1	--	--	--

TABLE A-21
Ca^{++} Inhibition of PPFase with Varying (Mg^{++})
Primary and Secondary Data for 1/V vs. 1/(Mg^{++})
(Fig. 26)

Mg^{++}		(Ca^{++}) = 0.00		(Ca^{++}) = 0.0005 M			(Ca^{++}) = 0.0040 M		
Conc. (M)	0.01/(Mg^{++})	V	100/V	% I	V	1/V	% I	V	1/V
0.001	10.0	19.6	5.10	52.8	9.0	11.10	92.0	1.6	62.5
0.003	3.3	30.8	3.25	42.5	17.7	5.65	85.0	4.7	21.2
0.005	2.0	31.3	3.20	25.6	23.3	4.30	78.0	6.9	14.5
0.008	1.2	31.3	3.20	--	--	--	--	--	--
0.010	1.0	28.6	3.50	10.9	27.5	3.64	58.5	11.8	8.5
0.014	0.7	25.6	3.9	9.1	23.3	4.30	--	--	--
0.018	0.6	23.2	4.3	--	--	--	--	--	--

TABLE A-22
Ca^{++} Inhibition of PPFase with Varying (ADP)
Primary and Secondary Data for 1/V vs. (Ca^{++})
(Fig. 27)

Ca^{++}	(ADP) = 0.00050 M			(ADP) = 0.00093 M			(ADP) = 0.00133 M			(ADP) = 0.00260 M***		
Conc. (M)	% I	V	1/V	% I	V	1/V	% I	V	1/V	% I	V	1/V
0.000	--	18.8	5.31	--	21.6	4.64	--	24.4	4.10	--	30.0	3.35
0.0002	12.7	16.5	6.05	10.7	19.3	5.20	7.4	22.6	4.44	0	30.0	3.35
0.0003	18.1	15.4	6.50	15.0	18.4	5.45	9.8	22.0	4.55	5.0	28.5	3.50
0.0005	22.4	14.6	6.85	18.0	17.7	5.65	14.2	20.9	4.80	7.6	27.7	3.61
0.0007	27.4	13.7	7.30	22.2	16.8	5.95	20.0	19.5	5.11	8.4	27.5	3.64
0.0010	33.8	2.5	7.40	34.0	14.3	7.00	28.5	17.5	5.71	21.0	23.7	4.21
0.0030	52.5	--	--	47.0	9.2	11.00	48.0	12.7	7.89	50.0	15.0	6.70
0.0050	72.3	5.2	19.20	--	--	--	65.0	8.6	11.60	61.0	11.8	8.50
0.0070	81.0	3.6	27.8	--	--	--	70.0	7.3	13.7	--	--	--

***Not plotted on graph

TABLE A-23
Optimum pH of PPFase**

Corrected final pH	Units of PPFase Activity
*5.56	6.4
*6.00	16.7
*6.62	21.7
*6.66	21.7
*6.69	22.2
*6.76	22.7
6.79	23.1
6.91	23.2
6.99	27.0
7.04	27.0
7.18	27.0
7.25	26.9
7.26	26.6
7.32	28.6
7.34	29.4
7.58	31.0
7.62	30.4
8.01	24.4
8.97	11.2

*Tris-maleate buffer used.

**Using 8.5×10^{-4} M PEPA.

TABLE A-24
K^+ Activation of PPFase with Varying pH
Primary and Secondary Data for 1/V vs. $1/(K^+)$

(K^+) final (M)	$1/(K^+)$	pH 6.01*		pH 6.86		pH 7.36		pH 8.11		pH 9.01	
		V	1/V	V	1/V	V	1/V	V	1/V	V	1/V
0.010	100	4.2	23.8	7.8	12.80	10.2	9.8	8.7	11.5	8.0	20.0
0.015	66.8	6.0	16.6	9.65	10.40	16.6	6.0	10.6	9.4	5.8	17.2
0.020	50.0	6.2	16.1	11.4	8.70	18.0	5.6	12.0	8.3	7.5	13.4
0.030	33.3	8.8	11.4	15.5	6.50	20.0	5.0	16.0	6.3	8.8	11.4
0.050	20.0	12.4	8.1	20.2	4.95	25.0	4.0	19.6	5.1	10.8	9.2
0.070	14.3	13.4	7.5	24.4	4.10	28.0	3.6	21.8	4.6	11.1	9.0
0.080	12.5	13.2	7.6	"	"	29.2	3.4	21.5	4.65	11.1	"
0.160	6.25	13.5	7.4	24.7	4.00	"	"	"	"	9.5	10.5

*Corrected pH's

TABLE A-25
Mg^{++} Activation of PPFase with Varying pH
Primary and Secondary Data for 1/V vs. $1/(Mg^{++})$

(Mg^{++})	$0.1/(Mg^{++})$	pH 5.24		pH 6.16		pH 6.70		pH 7.46		pH 8.04		pH 8.66	
		V	1/V	V	1/V	V	1/V	V	1/V	V	1/V	V	1/V
0.0010	100	2.7	37.5	7.5	13.4	11.0	9.0	17.9	5.6	21.7	4.6	15.9	6.3
0.0015	66.8	3.0	33.3	9.1	11.0	14.3	7.0	20.4	4.9	25.0	4.0	17.9	5.6
0.0020	50.0	3.1	32.2	11.2	9.0	17.2	5.8	25.0	4.0	27.8	3.6	17.0	5.85
0.0030	33.3	3.4	29.5	12.5	8.0	18.5	5.4	27.8	3.6	29.4	3.4	19.3	5.2
0.0050	20.0	3.4	29.5	13.8	7.3	20.8	4.8	23.2	4.3	26.4	3.8	--	--
0.0070	14.3	3.6	27.8	14.7	6.8	21.7	4.6	27.0	3.7	"	"	15.0	6.7
0.0080	12.5	3.5	28.7	15.8	6.4	22.2	4.5	27.8	3.6	24.4	4.1	12.8	7.8
0.0160	6.25	3.4	29.5	15.8	"	21.7	4.6	23.2	4.3	18.5	5.4	11.4	8.8

TABLE A-26
Variation of the Michaelis Constant with Varying pH
Primary and Secondary Data for 1/V vs. 1/(PEPA)

(PEPA) final (M)	0.01/(PEPA)	Corr. final pH 6.75		Corr. final pH 7.6		Corr. final pH 8.1	
		V	1/V	V	1/V	V	1/V
0.00010	100	10.0	10.0	5.3	18.9	1.23	81.0
0.00015	66.8	12.2	8.2	9.8	10.2	2.25	44.5
0.00020	50.0	15.1	6.6	12.5	7.4	4.3	23.2
0.00030	33.3	17.8	5.6	19.6	5.1	7.75	13.0
0.00040	25.0	19.0	5.3	23.2	4.3	10.4	9.6
0.00050	20.0	22.0	4.6	28.5	3.5	16.4	6.1
0.00070	14.3	23.0	4.4	29.5	3.4	22.5	4.5
0.00080	12.5	"	"	30.4	3.3	25.0	4.0
0.00100	10.0	23.3	4.3	"	"	27.0	3.7
0.00200	5.0	"	"	32.4	3.1	32.0	3.12